THE MECHANISM AND FUNCTIONAL CONSEQUENCES OF PASSIVE ACQUISITION OF MEMBRANE AND INTEGRAL MEMBRANE PROTEIN BY BOVINE POLYMORPHONUCLEAR NEUTROPHILS

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon

By

TYLER AUSTIN WHALE

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any materials in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5B4

ABSTRACT

In this Ph.D. dissertation, the capacity of cultured bovine polymorphonuclear neutrophils (PMNs) to passively acquire functional membrane proteins from apoptotic or necrotic cells was examined. The rapid transfer of membrane proteins from a variety of syngeneic, allogeneic and xenogeneic donor cells to PMNs was observed. In contrast to PMNs from other species, bovine PMNs did not express endogenous major histocompatability class II (MHC II) protein, either constitutively or inducibly. The entire bovine PMN population was, however, able to acquire detectable levels of surface MHC II or cluster of differentiation (CD) 3 protein following PMN co-culture with cells in conditions which permitted close contact with dieing cells. Therefore, it was hypothesized that membrane lipids and proteins were acquired by bovine PMN following fusion with microparticles (MPs) shed from either apoptotic or necrotic cells.

It was then determined whether the lifespan of bovine PMNs could be sufficient to provide an opportunity for PMNs to interact with T cells. Lymphocyte recruitment to sites of inflammation often occurs 3-5 days after the initial PMN recruitment. PMN survival would need to span this interval to provide an opportunity for an interaction between PMNs and lymphocytes. Pro-inflammatory cytokines, such as interferon (IFN)-γ and granulocyte macrophage colony stimulating factor (GM-CSF), and bacterial lipopolysaccharide (LPS) were observed to prolong the lifespan of cultured PMNs beyond 96 hours. These observations supported the conclusion that it was biologically possible for PMNs and T cells to interact at sites of inflammation.

Using confocal microscopy, direct evidence was provided for the formation and release of MPs from peripheral blood mononuclear cells (PBMCs) and the attachment of these MPs to bovine PMNs. A time-dependent integration of both MP membranes and integral membrane proteins into the PMN plasma membrane was also observed. The passively acquired membrane lipids and proteins then diffused throughout the PMN plasma membrane. Another observation was the formation of MPs which contained donor cell cytoplasmic proteins and subsequent transfer this cytoplasmic protein to recipient PMNs. These observations raised the possibility that MPs could also transfer genetic material. Thus, confocal microscopy provided direct evidence that MPs were one mechanism by which bovine PMNs could passively acquire membrane lipids and integral membrane proteins.

Finally, the functional consequences of passive acquisition of membrane proteins were examined using two different approaches. A significant increase in green fluorescent protein (GFP) transgene expression was observed following PMN infection using the GFP expressing bovine adenovirus vector (BAV304). These PMNs had passively acquired membranes from an adenovirus permissive cell line. This observation provided indirect evidence for the passive acquisition of a functional viral receptor protein. Direct evidence that PMNs passively acquired functional membrane proteins was provided by the observation that the passive transfer of ovine MHC II molecules to bovine PMNs enabled these cells to induce antigen-specific proliferation and cytokine expression by xenoreactive T cell lines. Despite a reduction in amplitude and duration, T cell responses induced by PMNs were qualitatively similar to those observed following activation by the

stimulator B cell line. These observations supported the conclusion that PMNs could function as antigen presenting cells (APCs) following the passive acquisition of MHC II protein.

In conclusion, this research project provided evidence that bovine PMNs have an impressive ability to acquire membranes and functional integral membrane proteins from dead or dying cells. The implications of this transfer of immunological information are discussed within the context of the role which PMNs might play in both innate and adaptive immune responses.

ACKNOWLEDGEMENTS

The completion of this thesis project represents the combined efforts from many different people. First and foremost, I extend my utmost appreciation to Dr. Philip Griebel. From the beginning to the end of this project, we have worked well together, making this educational experience entirely enjoyable and worthwhile. Philip showed unlimited enthusiasm and interest with my work, guiding me with creativity, open-mindedness and patience. The long hours he has dedicated to my development as a scientist have been invaluable, making me realize the important role and the huge impact an effective mentor can have on developing minds…a role that I will surely fulfill in my lifetime. His high quality of supervision and mentorship are also paralleled by his treasured friendship. Philip has made a true difference in my life.

Dr. Lorne Babiuk and Dr. Suresh Tikoo, along with my other committee members, Dr. Peter Bretscher, Dr. John Gordon, and Dr. Lydden Polley have all made substantial contributions towards my research project, both in time and helpful direction. Dr. Babiuk and Dr. Tikoo first introduced me to the Veterinary (now Vaccine) and Infectious Disease Organization (VIDO) in 1997 as an undergraduate co-op student. Their mentorship has also been very valuable in my scientific development.

I also received a great deal of assistance from various people along the way. Terry Beskorwayne and Yurij Popowych have provided me with exceptional technical help and guidance. The group in Animal Care: Dr. Don Wilson, Brock Evans, Barry Carroll,

Sherry Tetland, Carolyn McCormack and Jaime Maimer have provided a great deal of assistance with all animal related experiments. Joyce Sander, your motherly warmth and advice, along with the help you provide for every new VIDO member are very much appreciated.

I would like to acknowledge the Canadian Institute for Health Research as well as the Poultry Industry Council for providing the funding necessary to complete the work presented here.

A few special and key acknowledgements remain. To Dr. Christine Zink and Dr. Bill Black, two fabulous people and friends who had a significant and early impact on my road to becoming a scientist. My Grandfather, Dr. George Jones, has been an inspiration from as far back as I can remember, always challenging the norm and encouraging original thought, especially if it went against the grain. My parents Bruce and Deborah Whale, the two most incredible people I know, have provided me with all the support and opportunity a child could wish for. Finally, to my wonderful wife Julia Whale, the best result my scientific career will ever produce, has been remarkably patient, understanding and supportive throughout my PhD program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
ATP	Adenosine tri-phosphate
BAdV-3	Bovine adenovirus type-3
BAV304	BAdV-3 expressing green fluorescent protein (GFP)
BCR	B-cell receptor
CCR5	Chemokine receptor 5
CD	Cluster of differentiation
CD40L	CD 40 ligand
Cl2	Clone 2 B-cells
CLIP	Class II-associated invariant chain peptide
ConA	Concanavalin A
CpG	Guanosine-cytosine hexamer
CR1 and 3	Complement receptor 1 and 3
CTLA-4	Cytotoxic lymphocyte-associated antigen-4
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
DCFH-DA	2`,7`-dichlorofluorescein-diacetate
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
ER	Endoplasmic reticulum
FcR	Fragment crystallization receptor
FITC	Fluorescein isothiocyanate
fMLP	Formyl methionyl peptides
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GFP	Green fluorescent protein
GRO	Growth-related gene product
GP	Glyco-protein
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen - DR
HMGB1	High mobility group box 1
ICAM-1	Intercellular adhesion molecule-1
IFN-γ and α	Interferon-γ and α
Ig	Immunoglobulin
IL-1 through 18	Interleukin-1 through 18
LFA-1	Lymphocyte function associated antigen-1
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MDBK	Madin Darby bovine kidney
MEM	Minimal essential medium
MFI	Mean fluorescence intensity
MHC I, II	Major histocompatability complex I and II

MIP1 α and β	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
MOI	Multiplicity of infection
MP	Microparticle
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate,
NK	Natural killer
NSF	N-ethylmaleimide-sensitive factor
ODN	Oligodinucleotide
PAF	Platelet-activating factor
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PI-3 kinase	Phosphate inositol-3 kinase
PKC	Protein kinase-C
PKR	RNA-dependent protein kinase
PMN	Polymorphonuclear neutrophil
PMNp	Precursor (immature) PMN
PS	Phosphatidylserine
qRT-PCR	Quantitative RT-PCR
RAGE	Receptor for advanced glycation end products
RBC	Red blood cells
RNA	Ribonucleic acid
RT-PRC	Reverse transcriptase polymerase chain reaction
SA-FITC	Strept avidin-FITC
SEB	Staphylococcal enterotoxin B
SI	Stimulation index
SNAP	NSF-attachment protein
SNARE	SNAP-attachment protein receptor
SV40	Simian virus 40
TAP	Transporters associated with antigen processing
TCR	T-cell receptor
Th1 and Th2	T helper 1 and 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
t-SNARE	Target-anchored SNARE
TT	Tetanus toxoid
TUNEL	TdT-dependent dUTP-biotin nick end-labelling
v-SNARE	Vesicle-anchored SNARE

CHAPTER 1

LITERATURE REVIEW

1.1 Neutrophils

1.1.1 Introduction

Neutrophils, or polymorphonuclear neutrophils (PMNs) are phagocytic cells that participate in inflammatory reactions as a first line of defence against bacteria, fungi, parasites and viruses [1-3]. The recruitment of polymorphonuclear leukocytes or PMNs to an area of tissue injury is one of the primary cell responses during the initiation of host defence. Historically, the neutrophil has been regarded as a terminally differentiated effector cell. The principle role of the neutrophil was thought to be phagocytosis with an impressive assortment of preformed antibacterial systems and soluble mediators that provide an indiscriminate first line of defence against pathogens and tissue damage. This limited picture of a disposable cell responding blindly to instructions may not provide a complete view of PMNs.

PMNs are formed in the bone marrow at a rate of about 8 million per minute in humans, although some reports suggest a turnover rate 10 fold higher [4-6]. Following formation in the bone marrow, PMNs migrate to the bloodstream, and about 12 hours later move into the tissues. PMNs have the shortest half-life among leukocytes, with a half-life of around 35 hours before they undergo apoptosis, thus limiting their potential to participate in long lasting immunological interactions. PMNs constitute approximately 60-75% of the blood leukocytes in most carnivores, 50% in horses and 20-30% in healthy cattle, sheep and laboratory rodents [7].

1.1.2 PMN Recruitment

PMNs are the first cell recruited to sites of inflammation for several reasons; primarily because of their capacity to rapidly defend the host against invading microorganisms, and because they are readily available within circulating blood in high numbers. They exert their anti-microbial activity by three mechanisms that generally lead to the destruction of the pathogen: recognition and phagocytosis of the infectious agent, generation of toxic oxygen derivatives through the activation of NADPH oxidase, and the release of microbicidal molecules from their specialized lysosomes (called azurophil granules) and granules [8]. The various subsets of granules contained within the neutrophil constitute an important reservoir not only of antimicrobial proteins, proteases, and components of the respiratory burst oxidase, but also of a wide range of membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation. It is the controlled mobilization of these cytoplasmic organelles that permits transformation of the neutrophil from a passively circulating cell to a potent effector cell of innate immunity. Additionally, the regulated exocytosis of granules enables the neutrophil to deliver its arsenal of potentially cytotoxic granule proteins in a targeted manner, thus preventing widespread damage to host tissue in most situations. The contact between PMNs and microorganisms occurs frequently in tissues after a lesion or in organs directly exposed to the environment, such as the respiratory tract or the digestive mucosa. Under conditions of first contact between PMNs and microorganisms, PMNs are often in contact with infectious agents that are not covered by serum opsonins, such as antibodies and complement factors. PMNs, however, express certain non-opsonic receptors such as complement receptor 3 (CR3), CD14 [9] and all toll-like receptors except TLR3 (in

humans) [10,11], which are pattern recognition receptors that are able to recognize molecular determinants at the surface of microbes [12]. PMNs offer perhaps the most efficient line of defence against invading pathogens. Their immunological abilities continue to be expanded as we learn more about this cell type. Their crucial role, outlined by the fact that no animal can survive for more than a few days without PMNs, may be more diverse than previously thought [13]. PMNs are indeed a critical cell to call on as the first line of immunological defence.

Blood borne PMN are found in two main pools: a circulating pool and a pool sequestered in capillaries. During infection, the numbers of circulating PMN can increase 10-fold as they are released from the bone marrow and the sequestered pool. Previous studies have demonstrated that, in addition to certain potent chemotactic and proadhesive mediators for PMNs such as interleukin (IL)-8 and tumour necrosis factor-α (TNF-α), various peptides, polypeptides, fragments of polypeptides and lipids can cause the chemotaxis of PMNs. Specifically, formyl methionyl peptides (fMLP), C5a-derived from the fifth component of complement, and leukotriene B4 can all serve as potent neutrophil chemotactic factors [14]. However, if PMNs are to defend the host against microbial invasion, they must first leave the blood stream. Tissue specific recruitment of PMNs is mediated by changes in both the endothelial cells that line blood vessel walls and in the PMN following signals generated by inflammation. Bacterial products such as LPS or molecules (such as thrombin or histamine) produced by damaged tissues, cause capillary endothelial cells to express a glycoprotein called P-selectin (CD62P). Normally stored in granules, P-selectin moves to the cell surface within minutes after the cells are stimulated. P-selectin then binds to PMN expressed L-selectin (CD62L), triggering a rolling action that gradually slows the cells, allowing the second stage of adhesion to

occur. A lipid called platelet-activating factor (PAF), secreted by the endothelial cells, activates the rolling PMN which then express CD11a/CD18 or LFA-1. This is an adhesive protein or integrin that binds strongly to an inflamed endothelial glycoprotein called intercellular adhesion molecule-1 (ICAM-1; CD54), resulting in a complete PMN stop and attachment to the endothelial cell layer. PMNs are also activated by TNF-α and leukotrienes secreted by activated mast cells and secrete small amounts of elastase. The elastases cleave CD43, a protein shown by Woodman et. al. to have a dual role as a passive non-specific functional barrier to leukocyte-endothelial adhesion during non-inflammatory conditions, but during inflammation, CD43 also facilitates emigration of leukocytes into tissues [15], allowing an even stronger adhesion. Following further endothelial cell activation by IL-1 or TNF-α, the subsequent expression of E-selectin (CD62E) again enhances PMN adhesion. IL-1 also induces the production of the chemokine IL-8 (also designated CXCL8) from endothelial cells which attracts more PMNs, while TNF-α stimulates IL-1 secretion from endothelial cells. TNF-α also promotes vasodilation, procoagulant activity, thrombosis, and increases both expression of cell adherence proteins and production of chemotactic molecules. After adhering to blood vessel walls, PMNs emigrate into the surrounding tissues by squeezing between the endothelial cell layer and the basement layer. Since PMNs are the most mobile of all the blood leukocytes, they are the first cells to arrive at the site of inflammation.

This is a broad overview of PMN recruitment and function which does not include several other proteins which have more specific roles in PMN recruitment, adherence and migration.

1.2 Antigen Presentation

1.2.1 Introduction

Antigen processing and presentation pathways are central to the generation of adaptive immune responses [16]. The immune system needs to recognize target protein antigens from pathogens residing in both extracellular and intracellular locations. The crucial immune functions that direct and co-ordinate the delivery of the antigen for immune recognition and subsequent generation of T-cell responses have been recognized as components of antigen presentation pathways. These functions begin with proteins at various subcellular locations in the antigen presenting cell (APC), and end with the expression of a peptide-loaded major histocompatability complex (MHC) molecule on the APC surface, which can be recognized by T-cells of the appropriate specificity. This process involves intricate proteolytic processing events that generally allow for the separation of extracellular and intracellular pathogens into distinct antigen presentation pathways. However, recent evidence shows that this conventional dichotomy in the proteolytic processing of endogenous versus exogenous antigen is less restrictive than originally thought. The events that constitute the conventional MHC-restricted processing pathways are accompanied by interesting deviations that provide opportunities for antigens to cross over into their non-conventional processing pathways.

1.2.2 Adaptive Immunity

The successful establishment of adaptive immunity requires that antigen-specific lymphocytes undergo activation, expansion and differentiation. This process is determined to a large extent by the interaction between the T-cell and the APC, during which antigen-derived peptides in complex with the APC's MHC molecules are presented to the T-cell's antigen receptor (TCR) [17]. In order to function as an effective APC, a cell

needs to satisfy several criteria, including the capacity to: a) collect and cleave antigens; b) generate antigenic determinants; c) form stable complexes of the peptides with MHC class I or class II molecules; and d) express constitutive or inducible co-stimulatory molecules. To guide T-cell differentiation to a particular subset or phenotype, APCs also need to secrete cytokines, creating a milieu conducive for T-cell differentiation [17-21]. Virtually any cell expressing MHC molecules is a potential APC. However, APCs are not equal in their ability to stimulate T cells. The professional APCs, which include dendritic cells (DCs), macrophages and B cells, are better equipped than other cell types to maximize T-cell stimulation.

There has been an enormous amount of research done to determine the roles of the proteins involved in the processes mentioned above. Below is a brief outline of some of the major steps necessary in antigen presentation which have been discovered to date.

1.2.3 MHC I Restricted Antigen Presentation

The MHC I presentation pathway presents antigens from three principal sources: 1) peptide ligands generated as products of proteolytic degradation in the cytosol; 2) peptides generated within the endoplasmic reticulum (ER); and 3) some exogenous antigens that can be cross-presented on MHC I. Cytosolic peptides are first transferred to the ER via an ATP-dependent transporter associated with antigen processing (TAP) for binding to MHC I [22]. These peptides have already been degraded or cleaved into fragments by proteasomes and cytosolic amino peptidases and oligo peptidases. Once in the ER, they join other proteins in the process of translation. A few additional peptide degradation steps are required before an empty MHC I molecule is loaded and transported to the cell surface for recognition by cytotoxic T-lymphocytes (CTL).

Without going into great detail, suffice to say that there are various criteria that determine which proteins will be degraded within the cell to eventually serve as antigenic precursors. These include 'ageing' proteins, proteins with amino acid sequences which favour their degradation, proteins conjugated with ubiquitin or proteins that experienced errors during translation [23]. A high degree of stringency is required for the generation of peptides which can bind MHC I proteins as they have a closed binding groove. Unlike other peptide-binding proteins, MHC molecules bind peptide ligands as an integral part of their structure, thus making them unstable when peptides are not bound. This stable binding is important to prevent peptide exchanges from occurring at the cell surface which would prevent peptide:MHC complexes from being reliable indicators of infection [24]. Typically, MHC I peptides are octamer/nonamer sequences that have undergone distinct proteolytic events to generate exact –COOH and NH_2 termini. The binding of the peptide is stabilized at its two ends by contacts between atoms at the two termini, and invariant sites in the peptide-binding groove in MHC I molecules. As MHC molecules are highly polymorphic at certain sites along the peptide-binding cleft, this allows for broad peptide:MHC binding specificities. Each different peptide:MHC interaction will result in slight variations in conformation for both the peptide and the MHC molecule which in turn influence the binding of the T-cell receptor [24].

Exogenous proteins that enter the cross-presentation pathway have been shown to be degraded by proteasomes, and are thought to be dependent on TAP function [22], although TAP-independent pathways have also been implicated [25,26]. Although it is likely that phagocytosed proteins will eventually enter the ER for processing, interesting work has been done to show that ER membranes are an important component of phagocytic vesicles which acquire exogenous proteins for cross-presentation and

therefore, provides all the machinery necessary to present antigen on MHC I protein in an 'ER-independent' manner (independent to a degree as the ER membranes are still needed to fuse with the phagosome) [27]. Finally, both a TAP and ER independent MHC I presentation pathway have been identified, whereby proteins targeted to particular scavenger receptors are endocytosed and degraded within a proteasome. They are then loaded onto MHC I proteins which have also been endocytosed and dissociated from their existing peptide. The displaced peptide is replaced by the processed exogenous peptide [28]. Loaded MHC I proteins are then effectively being 'recycled' and used to present exogenous peptide. Many of these processes can also be modulated by the presence of certain cytokines that would be present at a site of inflammation.

1.2.4 MHC II Restricted Antigen Presentation

Following synthesis, MHC II molecules, in conjunction with an invariant chain protein, move to cellular exocytic compartments [29]. The invariant chain is cleaved by cathepsins, leaving a small peptide (CLIP) which must also be removed before processed peptide can bind to MHC II. The same proteolytic environment (proteosomes and low pH) which degrade MHC II destined proteins into peptides are also responsible for the removal of CLIP before MHC II/peptide binding can occur. There are three different pathways by which MHC II can be loaded with antigen. The classical pathway involves encountering peptides in the late endosomal-lysosomal compartments to which MHC II-CLIP has been transported [23]. Recycling of MHC II, similar to that described with MHC I, has also been observed, but unlike MHC I molecules, MHC II is more permissive to interactions with peptides of 'non-ideal' length as they have an open-ended peptide binding groove [30]. The third pathway involves presentation of cytosolic antigens, and

thus adds a new dimension to our understanding of antigen presenting pathways as now both MHC I and MHC II antigen presentation have access to both endogenous and exogenous antigens. This pathway has been found to be independent of TAP and the ER, but peptide processing involves cytosolic proteases [31,32]. Both the proteasome and calpain appear to play important roles in MHC II-restricted endogenous antigen presentation. In order for the processed cytoplasmic antigen to bind with MHC II molecules, they were transported into membrane organelles which contain unloaded MCH II protein [31]. Following translocation, peptides are further processed by acidic proteases within the endosomes, possibly even after binding to MHC II.

As mentioned previously, the dichotomy of antigen presentation is being reshaped into another biological phenomenon with significant redundancy in the classical and non-classical pathways. However, there is still a high degree of control within these systems and antigen presentation depends on several vital issues such as the form and identity of the antigen, intracellular localization, presentation by professional versus non-professional APC and the immunologic milieu (chemical signalling).

1.2.5 Cross-Presentation

Cross-presentation involves the uptake and processing of exogenous antigens within the major histocompatability complex (MHC) class I pathway. In 1976, Michael Bevan first published his observations that two cells with differing major histocompatability genes, but which shared the same minor histocompatability genes could cross-prime when only one of them was used to immunize a host, which recognized both of the major histocompatability proteins as self [33,34]. Although the concept of cross-presentation was still in its infancy, scientists quickly realized that the

acquisition of exogenous antigen by APCs, and presentation of this antigen by their MHC I proteins was the likely explanation for these observations. More than 25 years ago, Gooding and Edwards [35] implicated cross-priming by showing that SV40-transformed tumour cells generated SV40-specific CTLs restricted to the MHC haplotype of the host. Specifically, injection of allogeneic or even xenogeneic SV40 transformants into a mouse could confer subsequent resistance to a challenge with syngeneic SV40 tumour cells. At the time, they were unsure of the specific T-cell subsets generated, but we now know that indeed they were observing cross-presentation of SV40 antigen within host APCs on host MHC I, inducing host-restricted $CD8^+$ cytotoxic T-cells. The notion that membranes and proteins could be readily exchanged between immune cells was emerging, and the consequences were of obvious impact.

Cross-presentation is primarily performed by dendritic cells (DCs), which are not a single cell type but may be divided into several distinct subsets. Those expressing CD8α together with CD205, found primarily in the T-cell areas of the spleen and lymph nodes, are the major subset responsible for cross-presenting cellular antigens [36]. This ability is likely to be important for the generation of cytotoxic T-cell immunity to a variety of antigens, particularly those associated with viral infection, tumorigenesis, and DNA vaccination. At present, it is unclear whether the CD8α-expressing DC subset captures antigen directly from target cells or obtains it indirectly from intermediary DCs that traffic from peripheral sites [19].

Most cells have the capacity to present peptides on MHC class I molecules, a property that is particularly important for the identification of virus-infected cells by CTLs. Detection of virus-infected cells in this manner allows their destruction, thus limiting viral replication. To ensure only infected cells are killed, the MHC class I

pathway within most cells is restricted to processing endogenously synthesized proteins for presentation, excluding exogenously derived proteins. As a consequence, cells infected with virus can present viral antigens on their MHC class I molecules and be identified for CTL destruction, but bystander cells that merely endocytose viral debris cannot process such antigens to form MHC class I-restricted complexes and are therefore not targeted. For a few cell types, particularly DCs, the distinction between endogenous and exogenous antigen is not as strict, and MHC class I-restricted presentation of both endogenous and exogenous proteins can occur [37]. The unusual capacity to process exogenous antigens into the MHC class I pathway, referred to as 'cross-presentation', contradicts the 'direct' or 'classic' presentation route for endogenously synthesized proteins.

Although the major cell type known to cross-present antigens are the previously mentioned subsets of DC [36-38], several other cell types have been reported to cross-present, including B cells [39], endothelial cells [40], and particularly macrophages [41,42]. While cross-presentation by B cells and even endothelial cells might need further validation, substantial evidence argues that function occurs in both DCs and macrophages. Furthermore, several types of antigens have been reported to be cross-presented. These include soluble proteins, immune complexes, intracellular bacteria, parasites, and, most importantly, cellular antigens [19,36,41,43]. However, one question for which the answer is currently not fully elucidated is how cellular antigens enter the cross-presentation pathway.

Recent studies have implicated phagocytosis in cross-presentation [44], but several mechanisms have been speculated to lead to antigen capture, including uptake of apoptotic cells [45], nibbling of live cellular material [41,46], transfer of heat shock proteins

(HSPs) [47], and exosome uptake [48]. At present, there is limited *in vivo* evidence to categorically implicate any of these mechanisms. Capture of apoptotic cells by DCs has received a great deal of emphasis [49,50], with the likely false conclusion being that apoptosis is essential for entry of cellular material into the cross-presentation pathway. It is clear that apoptotic material can be cross-presented, as shown by the *in vitro* cross-presentation of apoptotic influenza-infected macrophages by human DCs [45]. However, it is far from certain that apoptosis is essential for access of antigen to the cross-presentation pathway as evidenced by the sampling of live cellular material by DCs [46]. Using live-cell imaging techniques, this group demonstrated that DCs (and primarily immature DCs) could acquire plasma membrane, and to a lesser extent intracellular proteins, from other DCs, macrophages, B-cells and activated T-cells in a cell contact dependent manner. Newly acquired antigens were cross-presented to MHC class I restricted T-cells, demonstrating the immunological relevance of such membrane protein transfer. These data are derived from in vitro studies, however, and the role of such a mechanism *in vivo* has yet to be confirmed.

What is certain regarding the mechanism of cross-presentation is that a phagosome to cytosol pathway for the translocation of exogenous peptide exists [51]. Recent work by Desjardins ***et. al.*** supports the hypothesis that phagosomes may have all necessary properties to support cross-presentation of exogenous antigens [44,52]. The finding that phagocytosis in macrophages proceeds by endoplasmic reticulum (ER) recruitment at the cell surface (a process referred to as ER-mediated phagocytosis), suggested that antigens from intracellular pathogens could have direct access, within phagosomes, to the ER machinery needed for MHC I presentation [53]. Using proteomics analyses, Desjardins succeeded in showing the existence within the phagosome of all the

necessary proteins required for cross-presentation. These results demonstrated that phagosomes were able to process exogenous peptides for MHC class I cross-presentation, extending the competence of phagosomes previously shown to be functional only for the processing of MHC class II complexes [54]. In addition, this work established that the use of ER, an organelle specialized in quality control, to form part of the phagosome membrane serves not only to minimize the utilization of the plasma membrane, but also confers properties allowing phagosomes to be fully integrated within the immune recognition system and play a direct role in cross-presentation.

1.2.6 Co-stimulation

In addition to MHC-peptide-TCR interaction (signal 1), T lymphocyte stimulation is not complete until a second costimulatory signal is received (signal 2). The costimulatory molecules B7.1 (CD80) and B7.2 (CD86) elicit both positive and negative costimulatory signals through the CD28 and cytotoxic lymphocyte-associated antigen 4 (CTLA-4) receptors, respectively [55,56]. The repertoire of costimulatory molecules continues to grow, revealing a careful orchestration of activities and many possible combinations of interactions. Interactions between adhesion molecules, such as leukocyte function antigen (LFA)-1 (on T cells) and ICAM-1 (on the APC) amplify the effects of the B7 molecules by anchoring the cellular interaction [57]. The cytokines IL-2, IL-7 and IL-15 are significant players in both the expansion and memory formation of $CD8^+$ T cells [58], whereas IL-12 elicits both IFN-γ production and T-helper function (Th1) through activation of $CD4^+$ T cells [59,60]. The complex interaction between an APC and a specific T-cell, which underlies T-cell priming and activation, entails the formation of a transient structure referred to as the immunological synapse [61]. The required molecular

composition of this immunological synapse varies with the state of T-cell development (naïve versus memory) and the specific type of T-cell (CD4 vs CD8 vs T-regulatory cell vs γδT-cell). Depending on the type of antigen however, co-stimulation may not be necessary. Some antigens called 'superantigens' for example, have been shown to require no co-stimulation in generating an immune response to them [62]. The stimulation of T-cells with a superantigen, however, is likely to indicate little about a cell's ability to present antigen, but rather the necessity to express MHC II protein. This is because instead of binding in the groove of the MHC molecule, superantigens bind to the outer surface of both the MHC II molecules and to several of the different V_β gene segments of the T-cell receptor [24]. As such, superantigens can stimulate a substantial percentage of T-cells (between 2-20% in mice and humans). This mode of stimulation, however, does not prime an adaptive immune response specific for the pathogen, but causes massive production of cytokines by CD4 T-cells, which can result in the suppression of the adaptive immune response [63,64].

1.2.7 Mixed Lymphocyte Reactions

Mixed lymphocyte reactions (MLRs) provide an *in vitro* system for determining T helper (T_H) cell proliferation in a cell-mediated response [24]. A two-way MLR is induced when allogeneic or xenogeneic leukocytes are co-cultured. This co-culture induces both T-cell populations to undergo extensive blast formation and cell proliferation during a primary stimulation and this response includes the generation of CTLs. Secondary stimulation of these alloreactive T-cells induces an extremely rapid and large proliferative response. A one-way MLR can be generated by rendering one of the stimulator populations unresponsive, often by using mitomycin C or a lethal dose of γ-

irradiation. The role of T_H-cells in a one-way MLR was investigated through the use of mAbs to block either MHC II or the T_H-cell CD4 protein. The MLR, and subsequent generation of CTLs, was abolished when either CD4, on responder T cells, or MHC II proteins, on stimulator cells, were blocked [65]. MAb blocking of MLR, combined with the observation that depletion of responder APCs had little effect on the MLR, supported the conclusion that responder T-cells directly recognized the genetically disparate MHC II molecules on the stimulator cells [65,66]. Thus, the basic components of an MLR include responder T_H-cells and allogeneic or xenogeneic stimulator cells which express surface MHC II with bound peptide. MLR reactions have also been observed in co-cultures which were MHC II compatible but MHC I mismatched. In these conditions, the primary proliferative response was significantly diminished but the induction of a response was interpreted as evidence that allogeneic APCs could provide sufficient co-stimulation to stimulate $CD8^+$ T-cells in the absence of T_H-cells [67].

An indirect T_H-cell stimulation pathway has also been reported whereby elimination of stimulator APCs did not result in complete abolishment of the MLR. That is, responder APCs were able to acquire stimulator-derived MHC II antigen, and present it to responder T_H-cells using self-MHC [67,68]. One group reported that this form of 'indirect' stimulation was unable to elicit a primary response, but was only able to stimulate primed T_H-cells [67]. Perhaps this limited T cell stimulation was due to the immunogenicity of the cross-presented antigen, or perhaps there was a relatively low number of responder T-cells which recognized foreign MHC peptides presented by self-APCs. The number of T-cells capable of recognizing foreign MHC molecules is thought to be between 1 to10% of the CD4 T cell population [24].

The MLR reaction, however, seems to be in contradiction to the experiments by Doherty and Zinkernagal [69], which illustrated the principles of MHC-restriction of T-cells. Their observation that cytotoxic T-cells were restricted in their recognition of virus infected cells expressing the same MHC genotype led to the concept of MHC restriction. They were fortunate to be investigating MHC I restriction, however, as an MLR involves the promiscuous recognition of MHC II/peptide by T-cells and would have given them results which argued against the concept of MHC restriction. It is now thought that such alloreactivity as observed in MLRs reflects cross-reactivity of T-cell receptors normally specific for a variety of foreign peptides bound by self MHC molecules. This cross-reactivity results, in part, because the spectrum of peptides bound by non-self MHC molecules on the transplanted tissues differ from those bound by the host's own MHC molecules, and the host's T-cells are not tolerant to these new peptide:MHC complexes [65,66].

1.3 A Potential Role for PMNs as Antigen Presenting Cells

1.3.1 Introduction

Although the most important roles of PMNs in innate host immune defence are likely to be the clearance of immune complexes, the phagocytosis of opsonized particles and the release of inflammatory mediators, the past twenty years of PMN research have presented the possibility that they are functionally much more diverse than previously imagined. Scientists also now recognize that for each leukocyte population there is a diversity of immune functions accompanied with significant redundancy and communication capabilities. That is, a specific immune function may be performed by several different cell types, perhaps with slight variations in efficiency or mechanism, but

with the outcome remaining the same. An example of such a redundancy in function is with APCs. There are multiple cell types all capable of specifically stimulating T-cells, albeit with different efficiencies. Research has also provided evidence that PMNs may have the capacity to fulfill all the necessary requirements to be considered APCs.

1.3.2 Antigen Acquisition and Processing

Tissue damage, by way of injury or infection, is followed by the release of vasoactive and chemotactic factors, recruiting PMNs to the site of inflammation. Being active phagocytes and expressing the Fc receptor (FcR) on their surface, PMNs can efficiently collect antigens by phagocytosis and FcR-mediated internalization [70,71]. PMNs can also recognize antigens by Toll-like receptors (TLRs). These receptors recognize microbial components and distinct nucleic acid motifs [72] and at least in humans, PMNs are thought to express all TLRs except for TLR3 [10,11]. Complement receptor 3 has also been implicated in non-opsonic phagocytosis of certain bacteria [8]. Once inside the phagosome, proteins are degraded by a multitude of proteinase enzymes such as proteases and acidic hydrolases.

An alternative mode of antigen acquisition, particularly for PMNs, may be extracellular antigen processing, generating peptides that bind directly to cell surface MHC class II molecules on nearby APCs. A recent report demonstrated that IL-8-stimulated PMNs secreted and activated gelatinase B [73], a matrix metalloproteinase, which cleaves type II collagen, leaving the immunodominant peptides intact for binding to MHC class II molecules for presentation to T cells. PMNs were also shown to process exogenous bacteria and particulate antigens through an alternate MHC class I processing pathway for presentation of peptides to T cells that did not involve ER-Golgi transport or

cytosolic processing [74]. By inhibiting ER-Golgi transport and cytosolic processing using brefeldin A and proteasome inhibitors, this groups was able to show that antigenic processing could occur via vacuolar processing and subsequent secretion of the processed antigen which could in turn be presented by other APCs [74]. If PMNs expressed MHC II, it is conceivable that they could process and excrete antigen that would in turn bind to their own MHC II molecules for presentation to T-cells, although this pathway for MHC II dependent antigen presentation has not yet been explored.

1.3.3 MHC II expression

A requirement for PMNs to play a direct role as antigen presenting cells would be the expression of MHC class II protein, necessary for $CD4^+$ T-cell activation. Presentation of antigen to $CD4^+$ T-cells is usually associated with professional antigen-presenting cells, such as dendritic cells, macrophages, and B-cells which constitutively express MHC class II. However, other cell types, such as endothelial cells, T-cells and Langerhan cells, have been shown to present antigen to $CD4^+$ T-cells when MHC class II expression is induced [75,76]. It has now been clearly demonstrated that MHC class II expression on PMNs of mice, humans and goats is either inducible or constitutive [77-82]. This research (Whale et. al., 2005, submitted) has also shown that bovine PMNs acquire significant amounts of MHC II protein shed from the membranes of apoptotic or necrotic cells, but they do not demonstrate constitutive or inducible expression. Several researchers have shown that in vitro incubation of human PMNs with GM-CSF, IFN-γ, or IL-3 results in low-level expression of HLA-DR [81,82]. In addition, significant HLA-DR expression has been observed on human PMNs *in vivo* following administration of either GM-CSF or IFN-γ [78,79]. It was determined that a combination of GM-CSF, IFN-γ, and

IL-3 was the most effective way of stimulating HLA-DR expression, with GM-CSF being the most potent stimulator [79,83]. It was also observed that donor variability existed, in that high levels of class II could not be induced on PMNs from all donors [83]. A number of potential explanations for these observations exist, ranging from difference in ability for cytokines to signal (be it from different numbers of receptors or even the presence of class II inhibitors) to the potential that there is an important genetic component that may play a role in MHC, cytokine or receptor expression.

1.3.4 Antigen Presentation and Co-stimulation

The next logical question to address is whether or not MHC II expressing PMNs can induce antigen specific T-cell responses, thus confirming their ability to present antigen to $CD4^+$ T-cells and directly stimulate an immune response. A major concern, however, is the lack of necessary second signals to activate T-cells, now a generally recognized requirement for appropriate T-cell proliferation and differentiation [84-86]. Inappropriate T-cell activation may also induce apoptosis or anergy [24]**.** One research group found little or no expression of B7-1 and B7-2 on human neutrophil populations, regardless of their source or whether they were incubated with GM-CSF and IFN-γ [62]. As a consequence, PMNs were unable to induce T-cell responses to the tetanus toxoid (TT) antigen being tested. Without appropriate secondary signals, T-cell production of IL-2, which leads to T-cell proliferation, is absent. Despite this lack of secondary signal, PMNs were still able to stimulate an antigen specific T-cell population with staphylococcal enterotoxin B (SEB) (considered to be a superantigen) [62]. It has been previously reported, however, that superantigens can interact with MHC class II and T-cell receptors in a way that eliminates the need for second signalling molecules to be

present on the antigen presenting cell [87]. However, another team found that PMNs cultured with autologous serum, IFN-γ and GM-CSF, did indeed express MHC class II, CD80 and CD86 at the mRNA and protein level. These PMNs induced proliferation of TT-specific T cells in a MHC class II-restricted manner, albeit an expectedly low proliferative response was observed relative to TT-presentation by monocytes [88]. It should be noted, however, that the possibility exists that contaminating DCs or monocytes could affect their RT-PCR detection of mRNA, while the detection of surface proteins on PMNs would be unaffected. Therefore, the possibility remains that the PMNs had passively acquired the necessary antigen presenting proteins. This group did, however, address the potential for low numbers of mononuclear APCs in the enriched PMN population to affect antigen specific T-cell proliferation. Their results showed that the 1-2% of contaminating monocytes were not a significant contributing factor of T-cell proliferation. Therefore, the failure to induce an anti-TT T-cell response in the previously mentioned report could be a result of low levels of expression of co-stimulatory molecules that deliver the second signal to T cells. Another study that supports the observation that PMNs can present antigen reported that PMNs of patients with acute, active Wegener's granulomatosis, express MHC class II, CD80 and CD86, and similarly present antigens to T-cells in an MHC class II-restricted manner [89]. T-cell proliferation was induced in both peripheral T-cells as well as antigen specific T-cell populations only with autologous PMNs. There was considerably less stimulation with peripheral T-cells, being in line with the low frequency of antigen-specific precursors in the periphery, and the total stimulation by PMNs was only about 50% that induced using the same number of monocytes. It was, however, conclusive evidence for antigen specific, MHC-restricted T-cell stimulation.

Several of the discussed reports suggest that PMNs can express the required accessory molecules, such as intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1, in sufficient amounts to stimulate T-cell activation, even in the absence of B7-1 and B7-2. The significance of ICAM-1 expression is that it is an accessory molecule that acts by adhesion to LFA-1 on the T-cell to enhance the interaction between the T-cell receptor and the antigen/MHC complex [82]. Fisher *et.al.* [90] showed that human PMNs expressing transfected MHC II and ICAM-1 molecules could stimulate T-cells at 100-fold lower concentrations of staphylococcal enterotoxin A (SEA) bacterial superantigen than with PMNs expressing MHC II alone. They also showed that $CD4^+$ memory cell stimulation was much less dependent on accessory molecule expression than naïve T-cell stimulation. In addition, they were able to stimulate both naïve and memory T-cells using plastic adherent ICAM-1 and MHC II transfected PMNs, indicating that costimulatory signals mediated to the T-cells through LFA-1 can be delivered physically separated from the TCR:MHC II signal [90]. Human peripheral blood PMNs have also been shown to constitutively express a B7-1-like molecule that interacts with CD28, regulating T-cell function [91].

1.3.5 PMN Cytokine Production

Until recently, it was generally believed that mature PMNs had limited capacity to synthesize de-novo proteins, and played only a passive role in inflammation through phagocytosis and the release of preformed cytotoxic compounds [92]. Indeed, recent investigations have shown that PMNs can be induced to express a number of genes whose products lie at the core of inflammatory and immune responses. These genes include not only the constitutively expressed Fc receptors, complement components

(complement receptors 1 and 3; CR1 and CR3), MHC class I, cationic antimicrobial peptides and NADPH oxidase proteins, but also a variety of cytokines [including interferon (IFN)-α, IL-1, IL-6 and vascular endothelial growth factor] and chemokines [including IL-8, TNF-α, growth-related gene product (GRO), α-defensins, macrophage inflammatory protein (MIP)-1α and β, interferon-γ-inducible protein of 10kDa (IP10) and monokine induced by IFN-γ] [14,93-102]. Because these chemokines are primarily chemotactic for PMNs, monocytes, immature dendritic cells and T-cell subsets, a potential role for PMNs in orchestrating the sequential recruitment of distinct leukocyte types to sites of inflammation is likely to exist. Such an important immunologic function implies that PMNs play a direct role in both cellular and humoral immunity [92]. Table 1 outlines many of the immunologically relevant proteins expressed by PMNs and provides a brief description of their role in regulating pathophysiological processes.

Table 1.1. A summary of PMN-secreted cytokines and their effects on the T-cell response

PMN-secreted cytokines	Immunological effects on other leukocytes	References
IL-1	Executes proinflammatory functions and T-cell co-stimulation. IL-1 might favour Th2-type immunity	95
IL-6	Polarizes Th responses towards Th2-type immunity	96
IL-8	Chemoattracts and activates PMNs, promotes extracellular antigen processing by inducing gelatinase B release from PMNs. Also chemoattracts T-cells	14
IL-10	Suppresses Th1 subset and acts as an anti-inflammatory cytokine	101
IL-12, IFN-γ	Induce Th1-subset bias. IFN-γ induces MHC II expression on APCs and PMNs from some species and activates macrophages	99
MIP	Chemoattracts and stimulates macrophages, DC and T-cells (Th1)	99
TGF-β	Suppresses inflammatory reactions and induces tissue remodelling	101
IL-3	Induces MHC II on PMNs in some species	101
TNF-α	Induces MHC II on PMNs in some species, enhances CD86 expression on endothelial cells and supports inflammation	98
GM-CSF	Increases CR3, FcR, MHC I, MHC II and B7 expression, which take part in antigen acquisition, antigen presentation and T-cell co-stimulation	101
IP-10, MIG, I-TAC	Exert angiostatic activities on T-lymphocytes	101
GRO	Chemoattractant and activator of PMNs	99
α-Defensins	Chemoattractant for monocytes and T-cells	100

1.3.6 Other Considerations

Whereas the functional activation of PMNs in response to external stimuli has been widely investigated, the modulation of PMN survival is not well known. One major problem with accepting that PMNs can act as APCs is the fact that they are thought to be too short lived to allow for proper antigen presentation and subsequent T-cell activation. Now, mounting evidence supports the idea that the life span of PMNs in vitro can be extended [62,83,103], including our own unpublished observations (Chapter 5) where approximately 25% of bovine PMN survive longer than 96 hours in appropriate *in vitro* culture conditions (a cytokine milieu that might closely resemble that of an inflammatory site). In one notable study, PMNs in culture were found to rapidly die with a greater than 50% loss within 48 hours, and nearly 100% after 96 hours [104]. In contrast, PMNs

incubated with IL-1β, TNF, GM-CSF, G-CSF, IFN-γ or LPS showed a marked increase in survival time, in some cases extending their half-life more than three fold [103]. Prototypic chemoattractants, such as fMLP, C5a and IL-8 had no affect on PMN viability[103]. All of the inducers of PMN survival were shown to interfere with the physiologic process of apoptosis. Because mature circulating PMN are terminally differentiated, short-lived cells, incapable of proliferation or self-renewal, the regulation of PMN survival at sites of inflammation may represent, in addition to recruitment from blood, a major mechanism through which functional PMN can be accumulated. This would allow for prolonged PMN function, important for the regulation of host resistance and inflammation, and may represent a crucial permissive step to enable a defined set of cytokines and microbial products to activate gene expression and alter PMN function.

A remaining piece of evidence to support a potential role for PMNs as APCs is that the immature or precursor form of end-stage PMNs (PMNp) can acquire dendritic cell characteristics in the presence of certain cytokines [105]. Dendritic cells are known to be the most efficient and effective antigen presenting cell. By culturing human PMNp in the presence of GM-CSF, IL-4 and TNF-α, their maturation program can be drastically shifted such that they acquire virtually all the characteristic features of professional APCs. These features include the expression of several additional APC-related surface molecules, including the costimulatory molecules B7-1 and B7-2, and accessory molecule CD40 [105]. Surprisingly the DC-driven PMNp were shown to be at least 10,000 times more efficient in presenting soluble antigen to autologous T cells when compared to freshly isolated autologous monocytes. Certainly, with better culture conditions, mature PMNs might be driven to acquire similar DC characteristics, but since PMNp are

regularly seen in inflammatory conditions, they seem to be likely candidates for an effective neutrophil source of APCs.

1.4 Apoptosis and Necrosis

1.4.1 Introduction

Eukaryotic cell death has been classified into two types: necrosis and apoptosis [106]. Necrosis is considered to be the result of a gross injury to the cells (a pathological reaction). This type of cell death is a passive degeneration in which there is a loss of integrity of intracellular organelle structures and the cellular plasma membrane. There is a lack of control of cellular programs, and intracellular contents are released directly into the extracellular fluid. This type of cell death is defined as ‘‘accidental.’’ In contrast to necrosis, the initiation and subsequent progression of apoptosis is strictly regulated by a cellular genetic program, and has hence been called ‘programmed cell death’ [107]. In apoptosis, cells do not die passively but rather actively kill themselves. It is a physiological process that is followed by the clearance of dead cells via phagocytosis by macrophages [108,109].

The term ‘‘apoptosis’’ was first coined by Kerr et al. [110], who described it as a cell death with a ‘‘characteristic morphology of hepatocytes which are dying under physiological conditions’’. These morphological changes include a pronounced decrease in cell volume, modification of the cytoskeleton resulting in significant membrane blebbing, a condensation of the chromatin, and degradation of the DNA into oligonucleosomal fragments [111]. Following these morphologic changes, an apoptotic cell sheds tiny membrane-bound apoptotic bodies containing intact organelles. These are thought to be quickly phagocytosed such that their intracellular contents are not released

into the surrounding tissue, and thus avoiding the induction of localized inflammation. While ‘‘apoptosis’’ was originally defined morphologically, ‘‘programmed cell death’’ comes from a functional definition of cell death that contrasts necrosis (accidental cell death). However, apoptosis and programmed cell death have become synonymous because the cells dying by programmed cell death usually exhibit the morphological characteristics of the originally defined ‘apoptotic’ cells.

1.4.2 Apoptotic Pathways

There are two major apoptotic pathways that have been well characterized: the mitochondria dependent intrinsic pathway and the death receptor mediated extrinsic pathway [107,112]. The former pathway is initiated by an internal sensor signal (such as the tumour suppresser protein p53), while the latter is triggered by external stimuli (such as Fas ligand). A group of cytosolic caspases are involved in initiation, execution, and regulatory phases of these pathways. The fundamental components of the intrinsic pathway are conserved among eukaryotic species, from invertebrates to mammals [113], suggesting that it might be the principal pathway for apoptosis induction. Finally, both intrinsic and extrinsic apoptosis are rapidly induced in the absence of de novo protein synthesis, indicating that all of the proapototic components in these pathways are preformed inside cells. Although some viruses were reported to induce apoptosis through the extrinsic pathway [114], the major pathway of most virus-induced apoptosis is likely to be through the intrinsic pathway. In response to the cellular stresses caused by viral infection, activation of p53 or an equivalent protein mediates the initial phase of apoptosis. This is followed by the sequential activation of caspases and regulatory components of the execution phase. Caspase 3 plays a key role in this phase, inducing

apoptotic cell death [115]. Alternatively, double stranded RNA-dependent protein kinase (PKR) is possibly involved in the induction of apoptosis by some viruses. PKR is an interferon-inducible enzyme and is required for the antiviral activities of IFN to become effective. During viral infection, PKR is activated and initiates a Fas-associated death domain (FADD)-dependent apoptosis cascade [116]. In addition to these two pathways, the massive accumulation of protein molecules and abnormal folding in the ER generates significant stress (termed ‘‘ER stress’’) on cells, resulting in the induction of a third form of apoptosis. Thus, abnormally upregulated synthesis of viral proteins in infected cells suggests that this ER stress pathway may also have a role in certain types of virus induced apoptosis [116].

Shortly after defining the morphological characteristics of apoptotic cell death, Wyllie et al. [117] reported that apoptosis is accompanied by a fragmentation of chromosomal DNA into oligonucleosome-sized DNA. Thus, the classical definition of apoptosis has been the cell death in which dying cells exhibit (1) the characteristic morphology (the shrinkage of cell volume, the perinuclear condensation of chromatin, and the fragmentation of the cell nuclei) and (2) the fragmentation of chromosomal DNA. This characteristic fragmentation of chromosomal DNA clearly suggests a massive alteration of chromatin structure during the apoptotic process. This alteration would accompany hypoacetylation of chromatin components and make high mobility group box 1 (HMGB1) protein stably associated with chromosomes. HMGB1 protein, a highly conserved cellular protein, is known as an abundant nonhistone chromosome-binding protein and also as a secreted protein. When released from cells, it can bind with high affinity, to the receptor for advanced glycation end products (RAGE) of macrophages and powerfully mediates inflammation (a type of ‘‘danger signal’’) [118,119]. In apoptosis, the

stable association of HMGB1 protein with chromatin can prevent it from being released into the extracellular fluid of the dying cells [120]. In contrast, disintegration of cellular structure in damaged or necrotic cells leads to the passive release of HMGB1 protein and the induction of inflammatory responses [120]. In addition, another biologically important characteristic of apoptotic cells is the exposure of phosphatidylserine molecules on the surface of the dying cells [121]. These phospholipid molecules usually appear on the inner surface of the plasma membrane but move to the outer surface when apoptosis is induced in infected cells. Phosphatidylserine molecules can function as phagocytic signals for macrophages [122].

1.4.3 Apoptotic:Necrotic Cell Ratio

Although apoptosis has been considered to be a separate process from necrosis, it has been observed that under conditions leading to massive apoptosis, considerable numbers of necrotic cells were also present [123]. Apparently, virus-triggered proapoptotic stimuli can also cause death by necrosis within some of the infected cells. The ratio of these two types of cell death is dependent on the physiological status of the cells upon infection. The possibility that these necrotic cells might also be a result of programmed cell death has also been proposed following observations that some necrosis is not the result of passive cell death but a result of some uncommon type of programmed cell death, not involving ordinary apoptotic pathways [124]. Cells may die through a process of programmed cell death (death as a specific physiological response) without demonstrating the characteristic morphology of apoptosis or vice versa [125]. In addition, the co-presence of necrotic and apoptotic cells at the site of infection may have

physiological significance *in vivo*, suggesting necrosis is not always the result of accidental passive cell death.

1.4.4 Virus Induced Apoptosis

Although the role of virus-induced apoptosis is considered to be limited as a cellular defence mechanism against mammalian virus infection, its effectiveness in limiting viral replication *in vivo* is reflected by the fact that most viruses have acquired ways to overcome it, such as through rapid multiplication and expression of anti-apoptotic genes [123]. The accelerated induction of apoptosis by TNF with the resulting decrease in viral growth is also representative of the effectiveness of this host defence mechanism [126]. Another role of apoptosis in the host defence system is to express a phagocytic signal on the surface of infected cells [109]. These cells undergo phagocytosis with resultant antigen presentation by macrophages to CD4 T lymphocytes. This sequence of events is to be considered as the initial steps in the development of acquired immunity against pathogenic infection. In addition, the direct interruption of the intracellular multiplication and release of the virus occurs as a result of the phagocytosis of the virus-infected cells. It was proposed that the major role of virus induced apoptosis in the host defence system is to help phagocytes recognize and digest virus-infected cells, leading to the induction of the immunological reactions necessary for the establishment of virus specific immunity [127].

1.4.5 Effects of Apoptotic and Necrotic Cells on Macrophages

Recent studies on the role of apoptosis in the development of acquired immunity, although they do not discount the possibility that apoptosis may play a role in the

induction of certain kinds of immunity, revealed that apoptosis elicits a suppressive effect on the potential immune activity of phagocytic macrophages [128]. Recognizing the critical role of phagocytic clearance of apoptotic cells in the resolution of inflammation [109], Reddy *et.al.* hypothesized that macrophages (cells primarily responsible for the clearance of apoptotic bodies) must have a selective survival advantage over other cells to be able to fulfill this important role. As inflammation subsides, large numbers of recruited cells are competing for a diminishing supply of growth and survival factors, resulting in the induction of apoptosis for many of these cells [129]. However, rapid clearance of these apoptotic cells is essential in preventing further inflammatory problems and yet macrophages are equally susceptible to starvation as other cells [130]. Reddy *et.al.* went on to show that macrophages do indeed have a survival advantage when there is a deficiency of survival factors that is conferred directly by the uptake of apoptotic cells, independent of the source of apoptotic cells or the method of induction of apoptosis. This extended to two distinct macrophage populations; terminally differentiated peritoneal macrophages and inactivated proliferating bone marrow derived macrophages. From these observations, they concluded that the role of apoptotic clearance as a survival factor may generalize to other phagocytic cells as well. Moreover, they showed that not only was there maintenance of survival, but a parallel inhibition of proliferation in the macrophage populations by simultaneously activating the PI-3 kinase pathway and inhibiting the ERK1/2 pathways. It stands to reason that limiting proliferation would be a good strategy during a period of deficiency of survival factors. Interestingly, a number of microbial products or danger signals which activate the innate immune system, such as LPS or bacterial DNA, have also been shown to inhibit apoptosis and proliferation of macrophages [128]. Although apoptotic cells and danger

signals (including necrotic cells) both activate PI-3 kinase, they have the opposite effects on the ERK1/2 pathways. Perhaps the different signalling events induced by apoptotic cells versus necrotic cells (or danger signals) may underlie the divergent outcomes generated by antigen presented in the context of one or the other, namely tolerance or immunity respectively [83]. Since there are both necrotic and apoptotic cells at sites of inflammation, phagocytes are certainly receiving different instructions depending on which cell type they phagocytose and will therefore react accordingly. The induction of immunological responses requires the presence of various cytokines in addition to antigen presentation by macrophages. Importantly, apoptotic cells do not seem to produce cytokines. In contrast, necrotic cells, which induce deregulated immune reactions, can provide the cytokines necessary for the initiation and establishment of virus-specific immunity. In this sense, the presence of necrotic cells at the sites where apoptosis is prevalent has important physiological significance *in vivo*. It is possible that the amount of necrosis determines the degree of inflammation and the immune reactions that result from viral infection.

1.4.6 Other Effects of Apoptosis and Necrosis

Many cells at sites of inflammation are, or will become either apoptotic or necrotic for various reasons. As both of these forms of cell death have been shown to release microparticles (MPs) or small fragments of membranes which contain proteins [106,131], researchers have increasingly suspected MPs might continue to play an important immunological role even after the onset of death. Of major importance is the fact that the induction of apoptosis almost never results in inflammatory injury to surrounding tissues [132]. This remarkable feature of apoptosis occurs for several reasons. First, the cell

membrane of cells undergoing apoptosis remains intact until relatively late in the process of cell death [132]. Second, apoptotic cells express unique surface markers that permit their rapid recognition and ingestion by phagocytes [109]. Hence, as long as phagocytic clearance of an apoptotic cell occurs before breakdown of its cell membrane, none of its cytosolic contents will be released into the extracellular space. In this way, despite the billions of cells that die each day by apoptosis, tissues are protected from an otherwise harmful exposure to the inflammatory contents of dying cells. Moreover, uptake of apoptotic cells actively inhibits the release of proinflammatory mediators such as TNF-α by macrophages [133].

Considering the suppressive role of apoptosis on the immune system as outlined above, the presence of necrotic cells at sites of apoptosis is intriguing. Although massive necrosis induces extensive inflammatory reactions at the site of infection, it also suggests that necrosis might play a biologically important role in the induction of virus-specific immunity. The balance of apoptosis and necrosis is essential for the infected animal to achieve sufficient immunological protection against the invading pathogens without significant immunopathology. To understand the host-pathogen interactions *in vivo*, future studies on the early innate immune responses to viral infection and the significance of necrosis and apoptosis in these reactions are required. There are no reports which describe differing PMN function following the uptake of apoptotic versus necrotic cells, but PMNs are known to phagocytose both types of dying cells [24].

1.5 Membrane Transfer

1.5.1 Introduction

A growing field of study is emerging around the transfer of membranes between leukocytes. Initially, the main focus of membrane transfer concerned dendritic cells, where scientists had coined the term '*cross-presentation*' for the acquisition, and subsequent processing and presentation of exogenous antigens on MHC class I molecules. DCs have also been shown to acquire plasma membrane fragments and cytoplasmic proteins from live cells [134]. It is theorized that this novel form of antigen acquisition provides a mechanism whereby DCs can access antigen in the absence of direct infection, and DCs can then present the antigen to T-cells within secondary lymphoid tissues. It appears, however, that more and more situations are being recognized where membrane fragments, along with functional proteins, are being transferred to a variety of cell types and in many cases this passive transfer of proteins results in altered cell biology. Some scientists have hypothesized that the transfer of membrane fragments to leukocytes may serve to either expand and/or regulate an immune response [134].

The preservation of function by passively acquired proteins could have profound consequences on the ability of the recipient cells to interact with and regulate other immune cells. Immunologically relevant phenomenon might still be observed even if protein function was not maintained. For example, if non-professional APCs were to acquire peptide-loaded MHC II from professional APCs at a site of inflammation, they may then have the ability to transport such antigenic information to surrounding lymph-nodes where soluble antigen might otherwise be excluded [135]. This may provide the immune system with a very efficient system for sampling antigens in the periphery and to transport the antigen into secondary lymphoid tissues. With the reported ability of DCs [134] and B-cells [136,137] to physically remove surface antigen from live cells, the ability for

cells to disseminate antigenic information without the same cell being able to present this antigen may be functionally relevant. The dissemination of several other types of protein information, by the exchange of membranes and proteins between cells, is also possible. For example, the acquisition of adhesion molecules could alter cells' adherence properties, or acquiring cellular receptors could alter the types of molecules able to interact with the recipient cell. Alternatively, cells may acquire all the proteins necessary to present antigen, or perhaps they lack some important co-signalling function that would render them more likely to play a role in the induction of tolerance or the down-regulation of an immune response.

As with most biological systems, there are likely to be several levels of control that determine the consequences of membrane transfer. A simple example of such control is seen in macrophages. If macrophages phagocytose apoptotic cell bodies then their potency as APCs is reduced, but following the phagocytosis of necrotic cell bodies, their capacity to function as APCs is greatly enhanced [83]. Thus, the type of membrane acquired from donor cells can confer distinctly different functions upon the recipient cell.

It is certain, however, that the growing number of membrane and protein exchanges being reported indicates that this phenomenon could be of broad biological relevance and worthy of a great deal more research.

1.5.2 Protein Transfer Amongst Leukocytes

There are several examples of integral membrane protein transfer between immune cells. For example, T-cells have been shown to acquire MHC II/peptide complexes from APCs (as well as other molecules) in a process that requires T-cell activation [138]. This study also demonstrated intercellular exchange of MHC II/peptide

complexes with B-cells, dendritic cells, macrophages and basophils in an activation-independent manner. Several publications have investigated this phenomenon and have observed that the T-cells which acquire such complexes serve to down-regulate existing immune responses by a mechanism associated with the induction of tolerance [138-141]. There has also been evidence to the contrary, that MHC II expressing T-cells can mediate the proliferation of T-cells specific for the same antigen in a secondary culture [142]. Although this phenomenon is not yet well characterized, the consequences of such protein transfer could extend well beyond a transfer of immunological information that activates or down-regulates immune responses.

Natural killer (NK) cells have also been shown to actively capture target cell membrane fragments before they deliver their 'lethal hit' [143,144]. This phenomenon occurs via the immunological synapse and is controlled in an ATP-dependent fashion by Src kinase, calcium and PKC, and involves actin rearrangements [144]. Although researchers are unsure of all of the consequences of this membrane 'nibbling', it is thought to be involved in the activation of the NK cell along with an expansion of immune recognition. Tabiasco *et. al.* have shown that following trans-synaptic acquisition of the primary EBV viral receptor protein (CD21), NK cells become susceptible to EBV viral infection [145]. Recent electron microscopy of a CD8 T-cell/target cell immunological synapse has provided some indication as to how this membrane exchange may occur [146]. There was evidence of a physical bridge formed between both cells, thus allowing for lateral diffusion of proteins and lipids along the fused membranes, without the proteins changing their orientation. Although it remains unknown how the initial membrane fusion between the T-cell and its target occurred, or the mechanism of their dissociation, this observation starts to explain the frequent membrane/protein

exchanges at the immunological synapse. Tabiasco's work effectively demonstrated the acquisition of a functional protein by NK cells and this transfer had a significant biological impact by rendering NK cells susceptible to EBV infection.

B-cells are another leukocyte that uses the B-cell receptor (BCR) dependent immunological synapse to capture membrane proteins. Specifically, multiple surface immunoglobulins will bind to multivalent binding sites on intact, membrane bound polymeric antigens (including membrane anchored proteins) from both APC and non-APC. This type of multivalent binding of antigens to the BCR elicits microscopic clustering of these bound proteins in the formation of an organized immunological synapse and induces B-cell activation. If there is a high avidity bond between the BCRs and the antigen, B-cells can extract membrane-bound antigens from the host cell for subsequent processing and presentation to T-cells [136,137]. This emerging form of antigen acquisition calls our attention to the unique aspects of molecular interactions in synapses, and, amongst other observations, has interesting implications for the cellular trafficking of intact antigens and viral particles.

Continuous and passive synaptic transfers have also been observed in some leukemia cell lines [147]. This observed transfer was reported not to involve cytosolic molecules or exosomes, but required cell contact. Intercellular synaptic transfer has mostly been described between immunological conjugates comprising an effector cell and its target. This particular study demonstrated that it may also occur between autologous cells other than immune cells. Although no functional consequence was studied following such transfer, it was hypothesized that this transfer could facilitate the formation of tumour related synapses (between other tumour cells or lymphocytes), or it could contribute to intercellular pathways for sustaining activation of these cells. A

study published in 1994, however, showed vesicular shedding from leukocytes resulted in the passive acquisition and subsequent functional changes in recipient epithelial tumour cells [148]. The vesicles were shown to attach to the plasma membrane of the recipient cells, although the authors concluded that complete insertion into the plasma membrane was less likely than simple adherence of the vesicles to the surface, as the acquired proteins remained in segregated 'rafts' rather than diffusing throughout the recipient plasma membrane. Remaining as a protein raft could, however, be a property of such protein acquisition, despite membrane fusion. Specific protein subsets were also characteristic of the shed vesicles. For example, vesicles might carry CD4/CD8 proteins, or HLA-DR/CD8 proteins, but not both. Similarly, not all recipient tumour cells were able to acquire all vesicles. Following co-culture of donor and recipient cells, recipient cells exhibiting one, both or neither set of proteins were identified, suggesting that acquisition of a given protein required an interaction with vesicles in a receptor mediated fashion. Upon acquisition of the leukocyte proteins, tumour cells exhibited a significant increase in adhesion to endothelial cells. Furthermore, such acquisition was also associated with modulation (both increases and decreases) of phosphorylation of many recipient proteins. These results confirmed that surface characteristics of cells are not solely dependent on their endogenous plasma membranes, but may be significantly altered following passive acquisition of proteins. These observations lend further support to the hypothesis that protein carrying vesicles are biologically important structures.

1.6 Microparticles:

1.6.1 Introduction

Cellular microparticles (MPs), also referred to as microvesicles, are fragments shed from the plasma membrane blebs of virtually all cell types when subjected to a number of stress conditions, including apoptosis [131]. MPs have also recently been shown to be reliable indicators of cellular stimulation, activation or tissue degeneration. Due to the increasing evidence that MPs can interact with neighbouring or even distant cells, they have also been identified as true vectors in the transcellular exchange of biologic information.

1.6.2 Microparticle Formation

Membranes of normal mammalian cells are composed of an asymmetric distribution of their aminophospholipids as follows: phosphatidylserine (PS) and phosphatidylethanolamine are mainly sequestered in the inner (cytoplasmic) leaflet, whereas sphingomyelin and phosphatidylcholine constitute the majority of the outer (exoplasmic) leaflet [149]. When cells are exposed to apoptogenic stimulation, the results are a spontaneous collapse of such membrane asymmetry [121] (Figure 1.1). MP release is an integral part of the membrane-remodelling process in which the asymmetric distribution of constitutive phospholipids between the two sides of a cell membrane is lost. Since PS has procoagulant qualities, its translocation to the exoplasmic side of cell membranes had initially led scientists to hypothesize that the primary role for vascular MPs (particularly those from platelets) involves some sort of hemostatic control [150]. However, PS can also act as a recognition determinant to specifically link activated or apoptotic cells to phagocytes [151]. In cultured nucleated cells, the level of MPs released

into the supernatant has been shown to correlate with the degree of apoptosis and could potentially be used as an *in vivo* or *in vitro* indicator of cell death [152]. MPs have been shown to be very heterogeneous in size (0.05 - 1μm), protein and lipid composition and have also been shown to carry membrane proteins including adhesion protein complexes [153].

1.6.3 Cellular Interactions with Microparticles

There is increasing evidence that MPs are detected by cells of various types, and often in tissues distant from the site of MP release. MPs can interact with cells through

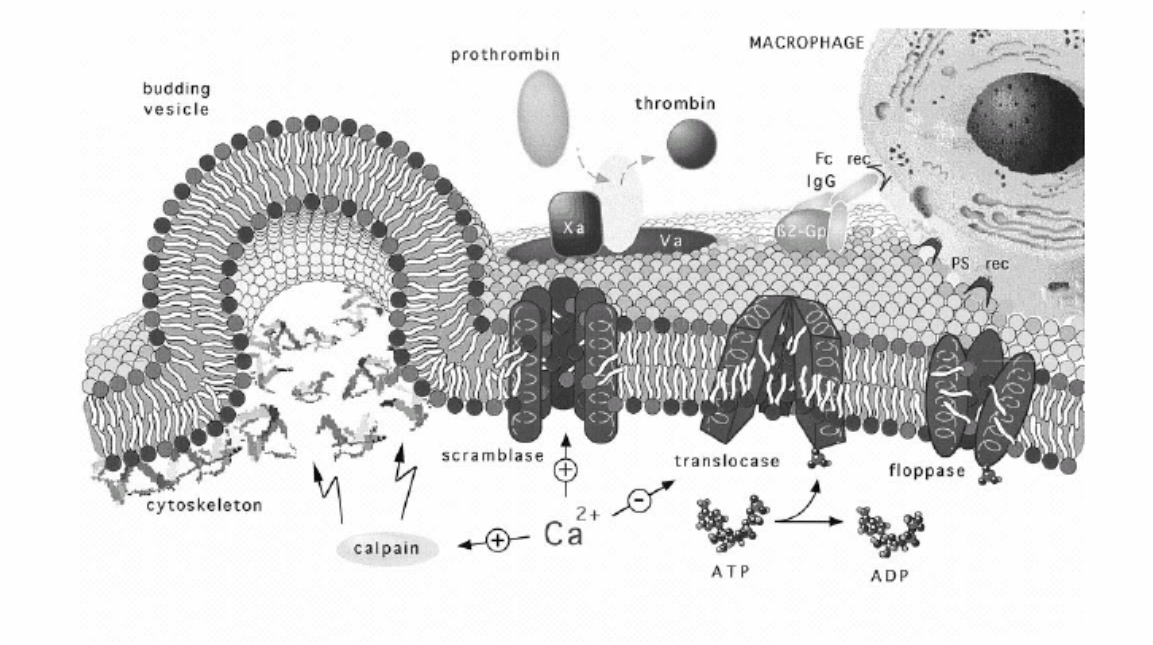


Figure 1.1 The regulation and physiology of membrane phospholipid asymmetry. This model describes how membrane phospholipid asymmetry is generated, maintained, and perturbed as a prerequisite to various phosphatidylserine-related pathophysiologies. Membrane lipid asymmetry is regulated by the cooperative activities of three transporters. The ATP-dependent aminophospholipid-specific translocase, which rapidly transports PS and PE from the cell's outer-to-inner leaflet; the ATP-dependent nonspecific lipid floppase, which slowly transports lipids from the cell's inner-to-outer leaflet; and the $Ca2^{+}$-dependent nonspecific lipid scramblase, which allows lipids to move randomly between both leaflets. The model predicts that the translocases are targets for $Ca2^{+}$ that directly regulates the transporter's activities. The figure shows that elevated intracellular $Ca2^{+}$ induces PS randomization across the cell's plasma membrane by providing a stimulus that positively and negatively regulates scramblase and translocase activities, respectively. At physiologic $Ca2^{+}$ concentrations, PS asymmetry is promoted because of an active translocase and floppase but inactive scramblase. Depending on the type of cell, elevated intracellular $Ca2^{+}$ levels can be achieved by cellular activation that generally results in the concomitant influx and accumulation of extracellular $Ca2^{+}$ and by its release from intracellular stores. Increased cytosolic $Ca2^{+}$ can also result in calpain activation, which facilitates membrane blebbing and the release of PS-expressing procoagulant microvesicles. Exposure of PS at the cell's outer leaflet. The appearance of PS at the cell's outer leaflet promotes coagulation and thrombosis by providing a catalytic surface for the assembly of the prothrombinase and tenase (not shown) complexes and marks the cell as a pathologic target for elimination by phagocytes. Recognition of the PS-expressing targets can occur by both antibody-dependent and direct receptor-mediated pathways [121].

protein surface receptors and in turn, transfer new proteins after membrane fusion [148]. For example, it has been shown that the chemokine receptor CCR5, which acts as a co-receptor for human immunodeficiency virus (HIV), can be passively acquired by non-CCR5 expressing cells when they fuse with CCR5 containing MPs. Such acquisition confers subsequent susceptibility to HIV infection [154]. Of significance was the observation of substantial transfer of CCR5 from PBMCs to endothelial cells during transendothelial migration which subsequently conferred HIV-1 tropism to $CCR5^+$ endothelial cells. This allows for free passage of blood-borne HIV to tissues via direct infection of the endothelial cell layer. Mack *et. al.* also reported that the transfer of CCR5 to monocytes, but not T-cells, was substantially reduced when monocytes were treated with cytochalasin D, an inhibitor of actin polymerization and phagocytosis. This could indicate that the incorporation of CCR5 containing MPs into monocytes involves an active process, whereas with T-cells, MPs were passively adsorbed to the cell membrane. Additionally, newly acquired CCR5 was internalized in monocytes following cytokine treatment. This observation could support the concept of a functional integration of CCR5 into the membranes of monocytes, or alternatively, a cellular process with the purpose of actively eliminating such newly acquired proteins from the surface of monocytes. With T-cells however, as CCR5 acquisition seems to be a passive process, they may lack certain control mechanisms as proposed for monocytes. The binding of MPs to different cell types can result in either integration into the recipient cell plasma membrane or merely adhesion to the plasma membrane. Different mechanisms of MP integration into host cell membranes may involve passive membrane fusion, endocytosis or phagocytosis, depending on the recipient cell type and its state of activation. Whether

or not MP proteins incorporate into the recipient cell membrane in the correct orientation, or at all, has obvious implications for the functional consequences of protein acquisition. Other examples of MP-related protein transfer are the transfer of arachidonic acid from platelet-derived MPs to other platelets and endothelial cells [155], and in 1986 is was reported that adherence of platelet-derived microparticles to monocytes was found to be responsible for 'false-positive' results in flow cytometry [156]. That is, in an attempt to detect monocyte derived glycoprotein IIb/IIIa (GP IIb/IIIa) expression using pulse-labelling with ^{35}S-methionine, researchers could only isolate unlabeled protein in the presence of significant numbers of platelets despite a positive flow cytometry analysis for GP IIb/IIIa surface expression on monocytes. Coupled with the fact that anti-GP IIb/IIIa antibodies failed to bind to isolated monocytes but bound to platelets, the researchers concluded that some sort of platelet contamination was responsible for the detection of GP IIb/IIIa on monocytes and implicated microparticles as the source of contamination.

Platelet MPs have also been shown to mediate leukocyte-leukocyte interactions [157]. This phenomenon may be able to assist in the transfer MPs between blood leukocytes, allowing cells of the immune system to preserve relevant immunological information, such as processed pathogen associated antigenic information, even after the onset of death.

1.6.4 Transfer of Genetic Information by Microparticles

There is also precedence for the transfer of genetic information through MPs. Tumour cell derived MPs have been shown to induce *in vivo* tumour formation through oncogene transfer to non-tumorigenic cells [158]. The same group also demonstrated that whole chromosomes or fragments thereof are horizontally transferred to neighbouring

cells by MPs generated from apoptotic cells. This observed gene transfer, however, may have implications beyond tumour progression. Horizontal transfer of genes has been reported in bacteria and fungi, playing an important role in the generation of antibiotic resistance and environmental adaptation [159,160]. Holmgren *et.al.* had also previously shown transfer of Epstein-Barr virus (EBV)-DNA along with genomic DNA to the nucleus of phagocytosing cells following the uptake of apoptotic cell blebs [161]. In this study, fluorescence *in situ* hybridization was used to show that up to 15% of phagocytosing cells had acquired EBV-DNA and subsequently expressed EBV-encoded genes at the protein and mRNA levels. It was also shown that HIV-DNA is transferred in a similar fashion to cells that are resistant to HIV infection, indicating a novel pathway of receptor-independent transfer of HIV [162]. The transfer of drug resistance genes has also been proposed to occur via uptake of apoptotic bodies [163].

All of the above mentioned observations lend credibility to the hypothesis that MPs carrying surface proteins, including MHC Class II, that are released from apoptotic leucocytes can adhere to and fuse with PMNs. Of greater interest is the MPs capacity to transfer proteins which alter cellular phenotypes, therefore modifying the intrinsic properties of the recipient cells. In light of the discovery of DNA transfer between cells (and subsequent expression), there remains a distinct possibility of RNA transfer between cells as well.

More investigations are required before it is proven that MP based transcellular exchange of biological information is a general characteristic with *in vivo* relevance. What is certain is that scientists should view MPs as functional biologic entities rather than simple cell debris.

1.7 Exosomes

1.7.1 Protein Composition of Exosomes

Exosomes are small membrane vesicles (30-100nm) of endocytic origin that are secreted by most cells in culture and are clearly distinct from the MPs that are produced by apoptotic cells [164]. Interest in exosomes has intensified after their recent description in antigen-presenting cells and the observation that they can stimulate immune responses *in vivo* [165,166]. Proteins contained in exosomes derived from particular cell types have shown a high degree of conservation, particularly those from antigen presenting cells [164]. Dendritic cell exosomes from both mice and humans for example, contained heat shock proteins, T-cell stimulation proteins (MHC I, MHC II, CD86), targeting and adhesion proteins, membrane fusion proteins and several other proteins of intrinsic interest to immune stimulation [164,167]. There is a particular abundance of MHC II in exosomes from all cells that express MHC II, as one example of exosomes from certain cell lineages containing distinct protein signatures. The protein composition of exosomes is even more limited when we consider that they do not contain any proteins of nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus origin. All of the exosomal proteins that have been identified are found within the cytosol, in the membrane of the endocytic compartments or at the plasma membrane [164]. Exosomes are not simply fragments of the plasma membrane, however, as they lack some abundant cell-surface proteins such as Fc receptors in DC-derived exosomes [167] or CD28, CD40L and CD45 in T-cell derived exosomes [168]. Finally, because exosomes form by inward budding from an endosomal membrane, they contain cytosol and expose the extracellular domain of transmembrane proteins (Figure 1.2). Much like with MPs, they also have a higher level of PS on the outer exosomal surface, supporting the hypothesis that all such vesicles

generated by reverse-budding (as with exosomes and microparticles) require a common cytosolic machinery [167]. In lieu of such findings, exosomes are now being considered a *bona fide* secreted sub-cellular compartment, and the current working hypothesis is that they have some, as yet unknown, physiological function, rather than primarily an alternative way of eliminating unwanted, non-degradable proteins. Perhaps, like exosomes, MPs might also selectively carry certain proteins more so than others, and can therefore conceivably transfer only a specific subset of proteins to a recipient cell.

1.7.2 Exosome Function

If such exchange of membrane, protein and cytosol occurs between exosomes (or other secreted membrane vesicles) and recipient cells *in vivo*, it could provide an organism with a mechanism of acquiring protein information and the resulting functional consequences that might otherwise be unachievable. Such vesicles could bear combinations of ligands to properly engage cell-surface receptors, similar to what happens when two cells interact, but without the need for direct cell-cell contact. Exosomes might simply bind to target cell membranes, endowing them with new surface molecules and, amongst other effects, could change the recipient cells' adhesion properties. Alternatively, exosomes might fuse with target cells thereby exchanging membrane proteins and cytosol between two cell types. Whatever sort of 'intercellular exchange' takes place, it is reasonable to assume that such a process would be strictly regulated, as with all other forms of intercellular communication. There are now numerous *in vitro* studies published which have examined the function of exosomes produced by APCs. In a few studies, exosomes have been shown to specifically stimulate T-cells [166,169], although with 50-100x less efficiency than viable APCs. Other experiments demonstrated tumour rejection

following immunization with exosomes produced from DCs pulsed with tumour antigens [165]. The potential remains, however, that such acquisition of antigen by DCs could induce either T-cell priming or tolerance. There is evidence of small vesicles produced in rat epithelial cells (named 'tolerosomes' as they were not properly characterized as exosomes) that have induced some degree of antigen-specific tolerance [170]. Therefore, the proposed role for exosomes in the immune system, with regards to the kind of immunological response they might induce, could depend on their cellular origin, the target cells that they encounter, and the immunological context of such interactions (inflammatory vs non-inflammatory environment). If a pathogen is taken up in the periphery by immature or maturing DCs (and possibly other APCs or leukocytes as well), they could secrete peptide-MHC complexes via exosomes endowing recipient APCs with the ability to stimulate specific T-cells to the newly acquired antigen. Alternatively, they could exchange exosomes after migration to the draining lymph nodes, thereby amplifying the magnitude of an antigen specific immune response due to the increased number of APCs expressing a particular antigen. In the absence of inflammation, such exchange could contribute to the induction of tolerance in the periphery [164]. There are also preliminary reports of antigen-independent effects of exosomes, primarily cell activation leading to cytokine production [171] or immunosuppressive effects [172].

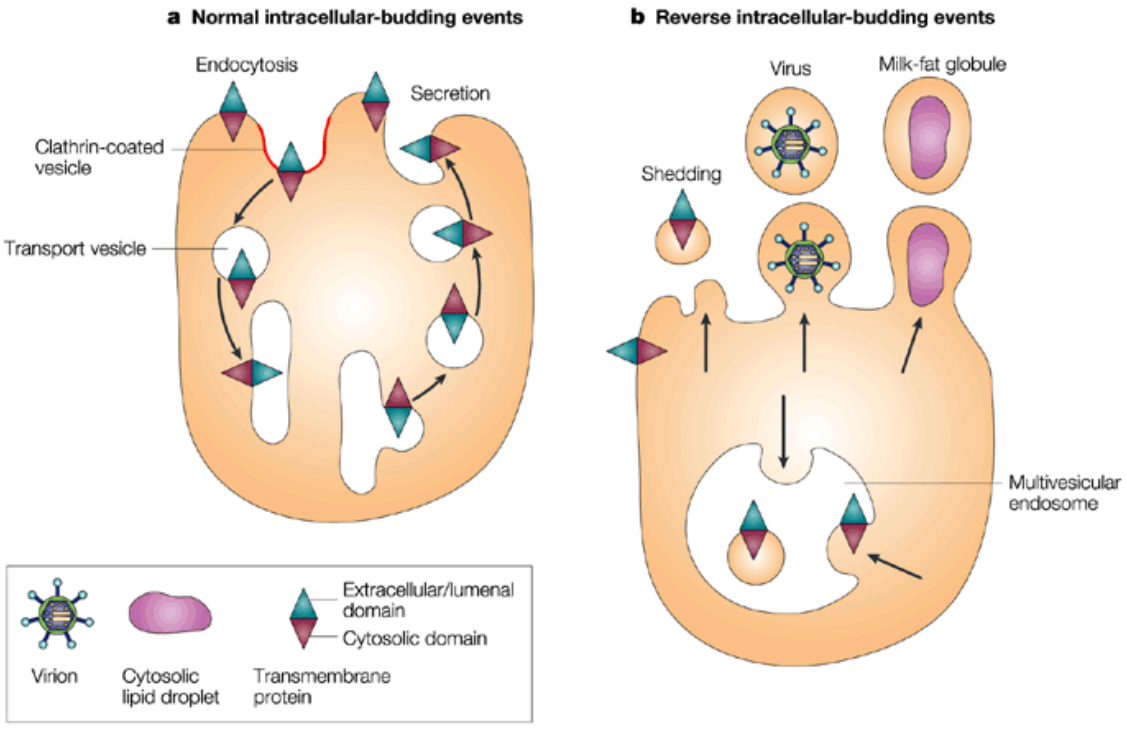


Figure 1.2 Orientation of various budding events occurring in a cell. **a** | Normal budding events. The transport of cargo proteins between intracellular compartments occurs by means of vesicles budding off a compartment and travelling through the cytosol towards another compartment (transport vesicles), or towards the cell surface (secretion). These vesicles contain proteins from the lumen of the donor compartment, and expose at their surface the cytosolic part of the transmembrane proteins that they carry. For the endocytosis of cell-surface receptors (clathrin-coated vesicles), vesicles that have the same membrane orientation form. **b** | Reverse budding events. Budding events in which the vesicle that forms contains cytosol and exposes the extracellular or lumenal domain of receptors can occur also. This is the case at the plasma membrane during virus budding, the secretion of milk-fat globules or the shedding of membrane vesicles, or during the formation of the internal vesicles of multivesicular endosomes [164].

In spite of the above mentioned studies, the physiological relevance of exosomes remains questionable. Although the experiments mentioned above were all carried out *in vitro*, there is also good evidence that exosomes are produced *in vivo* [173], and that they transfer MHC II protein to follicular dendritic cells which do not synthesize endogenous MHC II [174]. Much remains to be studied regarding this interesting phenomenon of membrane, protein and antigen sharing amongst leukocytes, to uncover the physiological role of this new form of cell-cell communication.

1.8 Membrane Fusion:

1.8.1 Introduction

A critical factor in the organization of phospholipid bilayers is the necessity of maintaining membrane integrity so as to prevent promiscuous membrane fusion. Conversely, the controlled fusion of intracellular membranes is essential for basic cellular functions, and the temporally and spatially regulated fusion of intercellular membranes is required for the formation of multicellular organisms. Little is known, however, about the molecular mechanisms involved in the fusion of multivesicular compartments or secretory lysosomes with the plasma membrane.

1.8.2 The Role of SNARE Proteins

Intracellular vesicle fusion that occurs in the secretory and endocytic pathways depends on a similar α-helical bundle structure as used by enveloped viruses during fusion with cell membranes [175]. In some cell types, fusion of endocytic compartments with the plasma membrane have been shown to be constitutive, whereas in other cell types fusion only occurs after activation in a Ca^{2+}-dependent manner [176]. After

recognition of vesicle and target membranes by the Rab guanosine triphosphatases and their effectors, most intracellular fusion events are determined by specific protein machinery which includes soluble factors (N-ethylmaleimide-sensitive factor, NSF, and soluble NSF-attachment protein, SNAP) and membrane complexes (such as SNAP-attachment protein receptor, SNARE) [177,178]. Both membranes that are involved in the fusion event need to bear specific SNAREs (known as vesicle-anchored or v-SNARE and target-anchored or t-SNARE), which are selectively localized to nearly all cellular compartments comprised of lipid membranes [179]. Particular v- and t-SNAREs are thought to dictate the specificity of intracellular membrane-fusion events, despite the fact that significant promiscuity of SNARE interactions has also been observed [177]. Prior to the SNARE interaction, there is an initial step that involves tethering molecules distinct from SNAREs [177]. Next, the v- and t-SNAREs bind each other in a pairwise, cognate fashion and interact to form a bundle of α-helices (the SNAREpin) that brings apposing membranes together and promotes their fusion [179](Figure 1.3). SNARE proteins must reside in opposite membranes for fusion to occur, but their pairing is spontaneous as assembly is favoured energetically. As such, cells and vesicles can bind together at 4^{o}C, mediated by SNARE proteins, and remain as unfused intermediates until cells are brought to physiological temperatures where the fusion process can be completed [179].

One important level of control then lies in the assembly of v-t-SNARE complexes and the kinetics of this process is further controlled by regulatory proteins such as members of the Rab GTPase family [180]. By modulating the rate of SNARE complex assembly at local sites either directly or indirectly, regulatory proteins can potentially add pairing specificity to different cellular compartments. Interestingly, it has been proposed that SNARES are themselves the minimal machinery needed for membrane fusion to

occur [179]. That is, they are both necessary and sufficient for membrane pairing and for lipid bilayer fusion. In addition, SNAREs are necessary as core transport machinery accounting for correct vesicle targeting to the principal cellular compartments. Other proteins found to be involved, such as SNAPs and NSF, are not essential for fusion after SNARE complexes pair between membranes, rather, their central role in these cases appears to be to separate v- from t-SNAREs after a round of fusion to allow for the next round [179]. They are generally regarded as necessary, however, in intracellular membrane fusion.

As our knowledge of membrane fusion progresses, scientists will endeavour to identify the many proteins and signalling pathways involved in regulating this process. Although the presently identified proteins are critical for membrane fusion and trafficking, it is probably that completely different membrane fusion mechanisms will be identified as a greater variety of cells are used for these investigations.

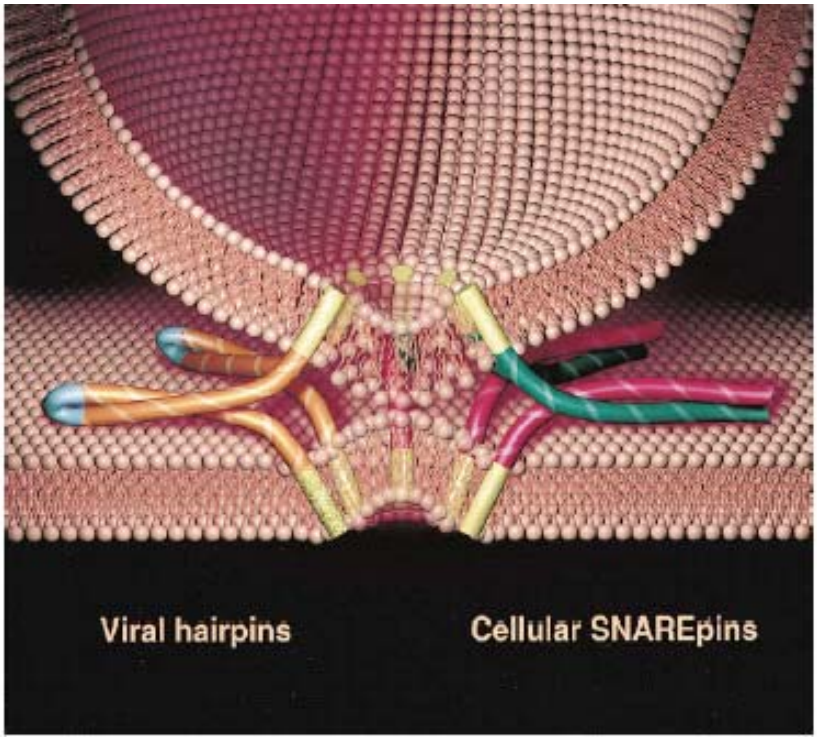


Figure 1.3. Contrasting viral fusion and SNARE mediated vesicle fusion. Cellular SNAREpins and Viral Hairpins v-t SNAREpins are complexes of cognate v-SNAREs (in green, in the transport vesicle above) and t-SNAREs (in red, in the planar target membrane below) bridging two membranes. Analogous t-t SNAREpins may also be possible. Membrane anchors are highlighted in yellow. The core of certain viral fusion proteins (at left, in orange) is diagrammed in a simplified fashion. The membrane anchor of the fusion protein (in yellow) is inserted into the viral membrane, represented by the spherical lipid bilayer. The fusion peptide (textured yellow) is inserted into the planar target membrane below. Viral fusion proteins generally consist of continuous polypeptides (indicated conceptually by the blue polypeptide loop), within which oppositely oriented (i.e., antiparallel) helical hairpin-like structures assemble in a helical bundle and are proposed to link up the two membranes for fusion [181-183]. A SNAREpin (whose precise internal structure is not yet known) consists of a 13–14 nm long helix-rich core rod of 2 nm width, which most likely contains the membrane-proximal helices of VAMP and syntaxin oriented parallel to each other [184,185]. In contrast to viral hairpins, cellular SNAREpins are formed from separate polypeptides that reside in different membranes before fusion. It is likely that multiple copies of viral hairpins or SNAREpins are needed to trigger fusion, and these are likely arranged in a ring-like structure at a contact point along the lines illustrated. The striking similarity between SNAREpins and viral hairpins suggests that extracellular and lumenally oriented viral fusion proteins, as well as intracellular membrane fusion proteins, all employ a fundamentally similar mechanism to coalesce lipid bilayers [179].

CHAPTER 2

THE RESEARCH OBJECTIVES AND HYPOTHESES

Within the last decade, it has become apparent that at least some cells have a remarkable capacity to function as donor or recipient cells for the exchange of membrane fragments and associated integral membrane proteins. The mechanisms of both donating and acquiring membranes and proteins can differ, and depends on the types of donor and recipient cells involved. Although the reasons and mechanisms for such transfer are poorly understood thus far, the potential exists for significant functional consequences in recipient cells.

The role of PMNs in adaptive immunity has also gained attention in recent years. Reports that PMN survival is prolonged following exposure to certain signals, the early recruitment of PMNs to sites of inflammation and their ability to migrate from blood to tissue and back to blood, and reports that PMNs can either endogenously express or passively acquire immunologically relevant proteins have lead to speculation that PMNs may play a role in both innate and adaptive immunity. Furthermore, PMNs may also be excellent candidates for disseminating immunological information throughout the body.

It was hypothesized that bovine PMNs could passively acquire functional proteins upon fusion with microparticles shed from apoptotic and necrotic cells. Enriched populations of bovine PMNs were used to evaluate the ability of this cell population to acquire a variety of integral membrane proteins and membrane fragments, with a particular emphasis on the transfer of MHC Class II protein from B-cells to PMNs.

The research objectives were divided into four main areas of investigation listed below along with the relevant hypotheses:

1) In Chapter 3, the hypothesis that PMN survival could be prolonged when cultured with immunologically relevant concentrations of certain cytokines and bacterial molecules was tested. PMN adherence to plastic was also monitored as an indication of PMN activation. Evidence for prolonged PMN survival *in vitro* could translate to the formation of relevant immunological interactions between PMNs and the later recruited lymphocytes to sites of inflammation *in vivo*. This would also allow for extended observation of PMN function *in vitro*.
2) A series of studies were designed to differentiate between possible endogenous expression versus passive acquisition of MHC II on bovine PMNs and to test the hypothesis that bovine PMNs passively acquired proteins and membrane fragments upon fusion with microparticles shed from apoptotic and necrotic cells. Based on these studies, a hypothetical model was proposed for PMN passive acquisition of membrane lipids and protein (Chapter 5).
3) Confocal microscopy was used to obtain direct evidence for PMN passive acquisition of membrane proteins and to analyse the mechanism by which PMNs acquired these membrane proteins. These analyses substantiated the hypothesis and the model proposed in Objective #2 (Chapter 6).
4) To test the hypothesis that functional proteins were acquired by bovine PMNs, two independent methods were designed to investigate the functional consequences of passive membrane protein acquisition by PMNs. The first method investigated the change in GFP-transgene expression following PMN acquisition of membranes from a BAdV-3 permissive cell line and the subsequent infection with the BAV304 vector constructed in Chapter 4. The

second set of analyses focused on the immunological consequences of passive acquisition of MHC II and the potential for PMNs to then function as APCs (Chapter 6).

CHAPTER 3

BOVINE POLYMORPHONUCLEAR NEUTROPHIL ACTIVATION AND SURVIVAL IN CULTURE

3.1 Introduction

It is generally accepted that the half-life of immature PMNs is only a few hours after leaving the bone marrow, and perhaps up to a few days for mature PMNs [7]. In vitro, PMNs rapidly die, with changes characteristic of cells undergoing apoptosis [104,186].

The brief lifespan of PMNs may be an important factor in limiting the potential role that PMNs might play in the propagation of an adaptive immune response. If, however, PMNs could survive for at least a few days, significant numbers of PMNs might co-exist with T-cells which have been recruited to a site of inflammation 4-5 days after pathogen infection. Alternatively, PMNs might interact with T-cells or professional APCs in the draining lymph nodes, where an extended period of survival would potentiate greater opportunity and range of interaction and subsequent immunological impact. It has been falsely reported that once PMNs have been recruited into the tissues, they do not return to the blood [103]. Several earlier studies provide evidence that PMNs are present in the afferent lymph, and have therefore exited the tissues to return to the secondary lymphoid organs [187,188] where they could possibly play a role in the control of the adaptive immune response. In addition, PMNs detected in efferent lymph must have originated from inflamed tissues and are destined to return to the blood [189,190].

The activation of PMNs in response to external stimuli has been widely investigated, but the modulation of PMN survival has not been well studied. There is however, mounting evidence that PMNs can be induced to survive for longer periods of time than previously believed [104,191]. For example, mature human PMNs incubated with various cytokines or bacterial products were shown to have a significantly longer half-life than non-treated PMNs. Specifically, IL-1 treated PMNs showed an increase in half-life from 35 hours to 115 hours. LPS treated PMNs were 95% viable at 72 hours of culture versus 14% viability for untreated PMNs. Mature circulating PMNs are terminally differentiated, short-lived cells, which lack the capacity to proliferate. Thus, regulation of PMN survival time at sites of inflammation may represent another mechanism by which PMNs could participate in the regulation of an adaptive immune response.

The aim of the present investigation was to test the hypothesis that PMN survival could be prolonged when cultured with immunologically relevant concentrations of certain cytokines and bacterial molecules by analyzing the effect of cytokines (GM-CSF and IFN-γ) and bacterial molecules (LPS and synthetic CpG-ODN, a synthetic analogue of bacterial DNA), known to be involved in the regulation of inflammatory reactions, on PMN survival. These cytokines were selected based on previous reports which suggest that both IFN-γ and GM-CSF are important pro-inflammatory cytokines to which bovine PMNs are known to respond [192,193]. LPS and CpG-ODN were selected as important representatives of bacterial molecules which induce innate immune responses in PMNs through TLRs. There is evidence that PMNs express both TLR4 (LPS receptor) and TLR9 (CpG-ODN receptor) and should therefore respond to these bacterial products [10,11]. The lifespan of bovine PMNs in vitro was analysed by quantifying the number of viable

cells at various culture time points. Viable cells were deemed to be those which excluded trypan blue dye and cells were counted directly with a haemocytometer. Similarly, using attachment to plastic as a sign of PMN activation, adherent cells were counted following culture with the same compounds.

3.2 Materials and Methods

3.2.1 Culture Media and Reagents

All cell cultures were maintained in AIM-V Serum Free Lymphocyte Medium (Gibco BRL, Burlington, ON) containing 20% (v/v) heat inactivated fetal bovine serum (FBS; Gibco BRL) and 50 μM 2-mercaptoethanol (2-ME; Biorad; Mississauga, ON), and unless otherwise indicated, cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. Recombinant bovine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) was from Genentech Inc. (San Francisco, CA), lipopolysaccharide purified from *E. coli* bacteria (LPS) was from Sigma-Aldrich (Oakville, ON) and recombinant bovine interferon-gamma (IFN-γ) from Ciba-Geigy (Basel, Switzerland). Synthetic CpG-ODN 2007 (QIAGEN, GmbH, Hilden, Germany) has been shown to have biological activity with cultured bovine leukocytes and the sequence and activity of this ODN has been previously described [194].

3.2.2 Purification of Blood Leukocytes

Bovine PMNs were isolated as previously described (Chapter 5)

3.2.3 Determining PMN Viability

Bovine PMNs isolated from blood collected from 5 animals (10 x 10^6 cells/well) were cultured in 6-well plates (Corning Inc., NY, USA) with different concentrations of FBS (2%, 10% or 20%), CpG-ODN (1μg/ml), GM-CSF (0.01ng/ml), LPS (50ng/ml), IFN-γ (1ng/ml) or combinations thereof (LPS + GM-CSF, LPS + IFN-γ, GM-CSF + IFN-γ and LPS + GM-CSF + IFN-γ) in a final volume of 6ml. All cultures containing the cytokines, LPS or ODN also contained 20% FBS. Cells were incubated for 24h, 48h, 72h, or 96h periods before being collected and counted. Each well was rinsed 3x with 2 ml calcium and magnesium-free phosphate buffered saline (PBSA; 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.3) to remove all non-adherent PMNs before adding 2ml of PBSA containing 0.1% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 2 minutes to remove the remaining adherent PMNs with vigorous pipetting. Viable and non-viable cells, as determined by trypan blue dye exclusion and inclusion respectively, were then counted using a microscope and haemocytometer, and the percentage of viable cells was determined by comparison to the total cell number. The relevant data was collected by comparing cell counts from a larger number of animals rather than performing several replicate treatments with fewer animals.

3.2.4 Measuring PMN Activation

Bovine PMNs (20 x 10^3 cells/well) were incubated in each well of polypropylene plastic 8-chamber slides (Sigma-Aldrich) with Aim-V/20% FBS either alone or with CpG-ODN 2007 (1μg/ml), GM-CSF (0.01ng/ml), LPS (50ng/ml), IFN-γ (1ng/ml) or combinations thereof (LPS + GM-CSF, LPS + IFN-γ, GM-CSF + IFN-γ and LPS + GM-CSF + IFN-γ) in a final volume of 300μl. After the 24h culture period, the chambers were removed, and the slides were submerged 3x in PBSA to remove any non-adherent

cells. The slides were allowed to air-dry before staining with DifQuick (Baxter Scientific, Miami, FL) and examined at 400x magnification under a microscope. Plastic-adherent PMNs were counted and recorded. One replicate per treatment for each of 4 animals was counted.

3.2.5 Statistical Analysis

All data presented were non-parametric in their distribution and comparisons between treatment groups were performed with Kruskal-Wallis analysis, using a Dunn's post-test (GraphPad Prism, CA, USA). *P* values less than 0.05 were considered significant.

3.3 Results

3.3.1 PMN Viability in *in vitro* Culture

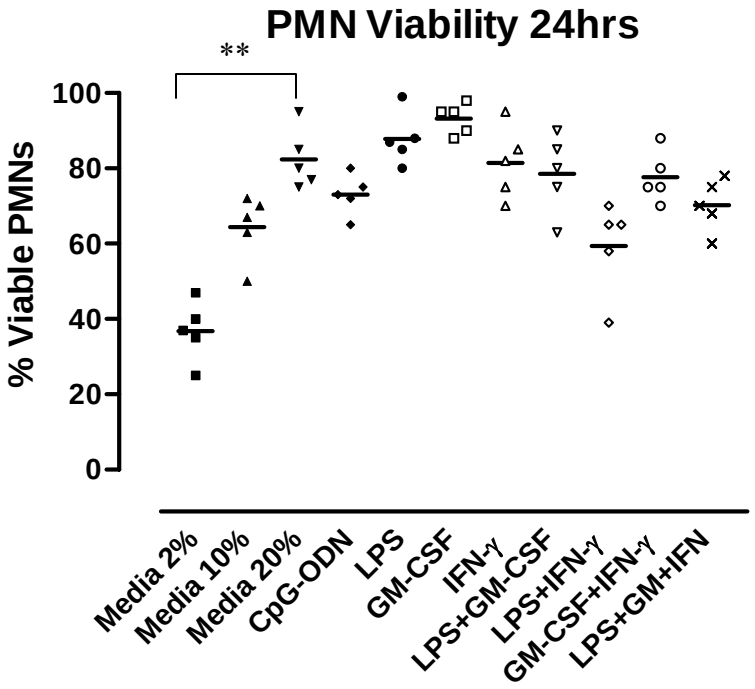
In order to consider bovine PMNs as a cell type with the potential to contribute to the induction of adaptive immune responses, they must survive long enough such that they have an opportunity to interact with lymphocytes recruited to sites of inflammation or the draining lymph node. The *in vitro* viability of purified bovine PMNs was analysed following culture with various serum concentrations along with the addition of recombinant bovine cytokines (GM-CSF and IFN-γ) and bacterial products (LPS, synthetic CpG-ODN 2007). Although all cultures began with 100% PMN viability, in the condition containing the typical culture media supplemented with 2% FBS, PMN viability declined rapidly with greater than 60% cell death over a 24 hour period (Figure 3.1A) and almost 100% cell death by 48hr (Figure 3.1B). Media supplemented with 20%

FBS on the other hand maintained significantly higher PMN viability as compared with media containing 2% FBS (Figure 3.1A-D). Of particular interest was the ability of LPS combined with either GM-CSF or IFN-γ (or both) to maintain nearly 30% viability during a 96 hour culture period (Figure 3.1D). In contrast, CpG-ODN 2007 did not prolong PMN survival either alone (Figure 3.1A-D) or in combination with other cytokines (data not shown). These experiments demonstrated that bovine PMN viability can be maintained for a prolonged periods (compared with media supplemented with 2% FBS) when cultured with media + 20% FBS or stimulated with combinations of LPS, IFN-γ and GM-CSF.

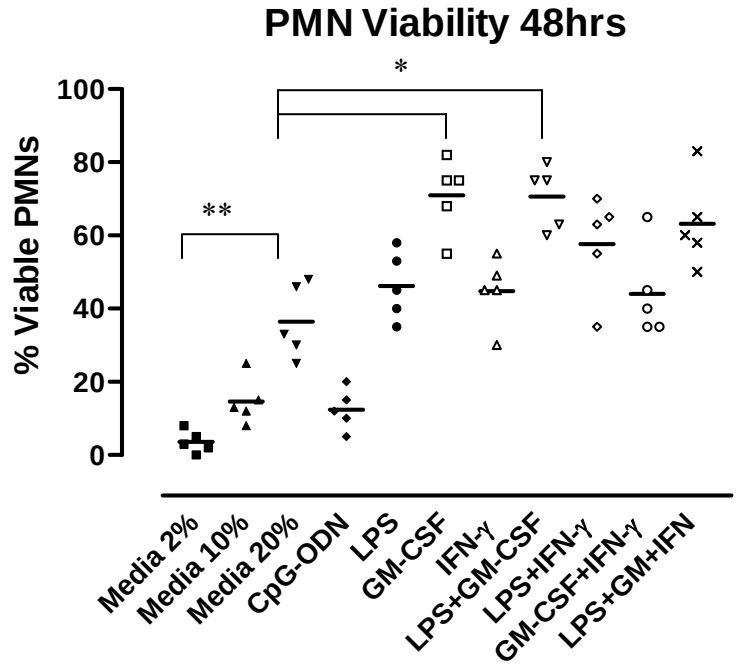
3.3.2 PMN Activation

The relationship between neutrophil survival and activation was also investigated by analysing the induction of neutrophil adherence to plastic within a 24 hour culture period. Following PMN culture with various combinations of cytokines and bacterial products (outlined above), there was a significant increase in cell adherence to plastic culture slides (Figure 3.2). Increased cell adherence was interpreted as a sign of neutrophil activation [24]. Consistent with the results for the prolongation of PMN viability, the combination of LPS and pro-inflammatory cytokines induced the greatest increase in adherent cell number. This experiment demonstrates that culturing PMNs

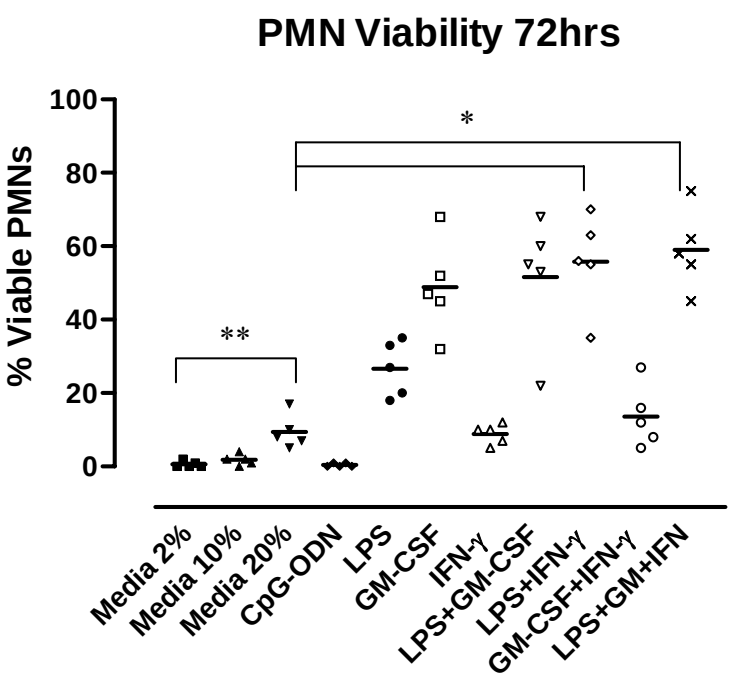
A


PMN Viability 24hrs
**
% Viable PMNs
100
80
60
40
20
0
Media 2%
Media 10%
Media 20%
CpG-ODN
LPS
GM-CSF
IFN-γ
LPS+GM-CSF
LPS+IFN-γ
GM-CSF+IFN-γ
LPS+GM+IFN

B


PMN Viability 48hrs
*
**
% Viable PMNs
100
80
60
40
20
0
Media 2%
Media 10%
Media 20%
CpG-ODN
LPS
GM-CSF
IFN-γ
LPS+GM-CSF
LPS+IFN-γ
GM-CSF+IFN-γ
LPS+GM+IFN

C


PMN Viability 72hrs
% Viable PMNs
100
80
60
40
20
0
*
**
Media 2%
Media 10%
Media 20%
CpG-ODN
LPS
GM-CSF
IFN-γ
LPS+GM-CSF
LPS+IFN-γ
GM-CSF+IFN-γ
LPS+GM+IFN

D

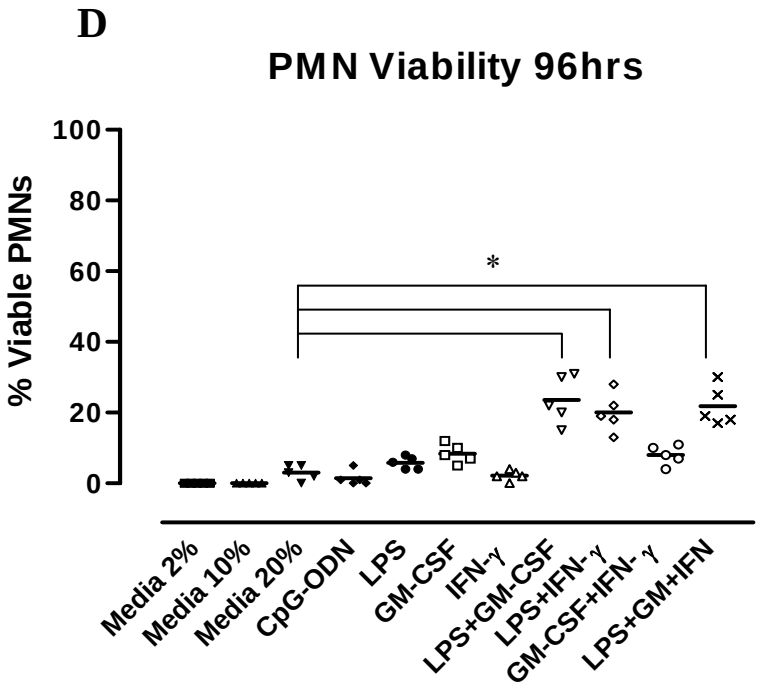

PMN Viability 96hrs
% Viable PMNs
100
80
60
40
20
0
*
Media 2%
Media 10%
Media 20%
CpG-ODN
LPS
GM-CSF
IFN-γ
LPS+GM-CSF
LPS+IFN-γ
GM-CSF+IFN-γ
LPS+GM+IFN

Figure 3.1. PMN viability during different culture conditions. 10 x 10^6 bovine PMNs from 5 different animals were cultured for (A) 24h, (B) 48h, (C) 72h, or (D) 96h with different FBS concentrations as follows; 2% (media 2%), 10% (media 10%) or 20% (media 20%). All remaining cultures were completed using 20% FBS with the addition of the following stimulants; 1μg/ml CpG-ODN 2007 (CpG-ODN), 50ng/ml LPS (LPS), 0.01ng/ml GM-CSF (GM-CSF), 1ng/ml IFN-γ (IFN-γ), or combinations (using the same concentrations) as follows; LPS + GM-CSF (LPS+GM-CSF), LPS + IFN-γ (LPS+IFN-γ), GM-CSF + IFN-γ (GM-CSF+IFN-γ), or LPS + GM-CSF + IFN-γ (LPS+GM+IFN). Viable PMNs were collected and counted by trypan blue exclusion at the end of each culture period.
*Significant difference between indicated culture groups (P<0.05), and ** (P<0.01).

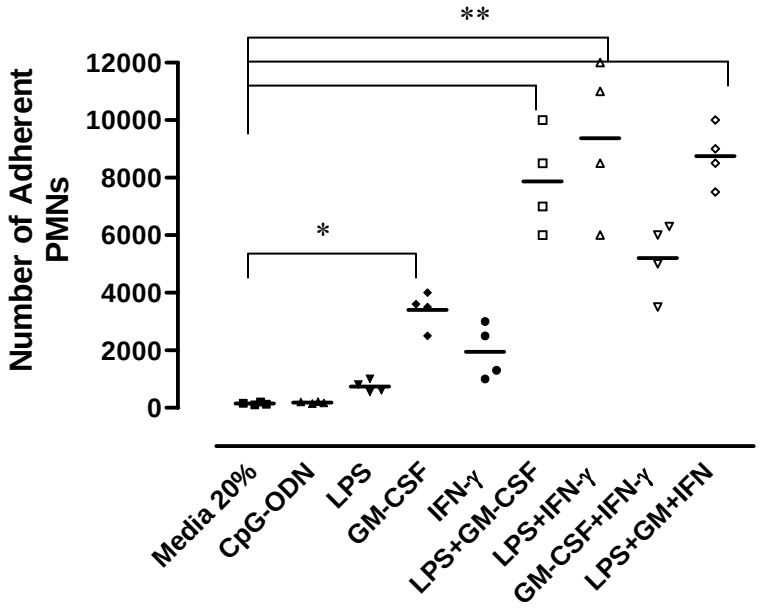


Figure 3.2. PMN adherence to plastic during different culture conditions. 20 x 10^3 bovine PMNs from 4 different animals were cultured for 24h using the same stimuli and 20% FBS as outlined in Figure 1. Adherent cell numbers were manually counted using a 400x magnification on an inverted microscope.
*Significant difference between indicated culture groups (P<0.05), and ** (P<0.01).

with combinations of LPS, IFN-γ and GM-CSF can significantly alter PMN adherence to plastic (as an indication of cell activation) and this response correlated with an increase in PMN lifespan.

3.4 Discussion

For PMNs to play a role in the induction of an adaptive immune response, they must be able to interact with T-cells at both the appropriate tissue site and within the time frame required for induction of T cell responses. There are several conceivable means by which PMNs could contribute to the induction and maintenance of an adaptive immune response, only some of which would necessitate prolonged PMN survival. For example, if PMNs are indeed capable of presenting antigen to T-cells, they could stimulate a secondary immune response in effector T-cells within minutes of a cognate interaction [24]. By comparison, if PMNs are to present antigen to naïve T-cells, they might need to survive for several days before T-cells are recruited to the site of inflammation or the draining lymph node. Alternatively, PMNs might perform a simple role in the dissemination of immunological information by transporting antigen to secondary lymphoid tissues, where other APCs could acquire and present this antigen. Finally, PMN contribution to the induction of an adaptive immune response might be very indirect through the secretion of immune modulating cytokines. Whatever their role, a longer period of PMN survival and activation would certainly enhance their opportunity to make a more significant impact on immune responses.

This group of experiments was performed to determine if there were biologically relevant conditions that could both prolong neutrophil survival while simultaneously inducing a state of activation such as would be expected before the production of

immunologically relevant proteins and cytokines. PMNs are known to be the leukocyte population with the shortest half-life of approximately 35 hours [7]. Their brief lifespan would appear to limit their capacity to interact with lymphocytes which may require several days before recruitment to sites of inflammation [24]. However, here we have effectively demonstrated that PMN survival can be prolonged (>96 hrs) if stimulated by the appropriate cytokines and pathogen-associated molecular pattern molecules which may be present at sites of inflammation. Simply increasing the concentration of FBS in the culture medium, from a typical cell culture level of 2% to 20%, increased PMN viability during a 24 hour culture period by over 40% (Figure 3.1A). Any further increase in FBS levels had little additional effect on PMN viability over time (data not shown). Of further interest was the increased level of viability when PMNs were cultured with low concentrations of cytokines, in particular GM-CSF alone or a combination of LPS with either GM-CSF or IFN-γ. The concentrations of cytokine and LPS used to stimulate the PMNs were titrated such that we observed a significant increase in PMN half-life and viability, with a minimum effective concentration of each stimulant. Lower concentrations of all of the stimulants used still increased PMN viability, but not to the same degree as the data presented. Furthermore, the cytokine concentrations used in these experiments were well within the range detected in tissues at sites of inflammation [195-199].

We encountered a difficulty with certain PMN collections at the 24hr time point. Specifically, many PMNs remained adhered to the surface of their culture plates, particularly with the LPS + IFN-γ treatment. Our attempts to remove such adherent cells with PBSA + 0.1% EDTA or even trypsin were successful at removing most of the adherent cells from all conditions not containing IFN-γ, but between 5-15% of the total

PMN population remained adhered in the 24hr cultures containing IFN-γ. Thus, the values reported for these conditions represent an under-estimate of PMN viability.

As cell activation is often characterized by the increased expression of surface adhesion proteins [24], the number of plastic-adherent PMNs following culture with the same stimulants used in the PMN viability experiment was measured. The 24 hour culture period was chosen since little difference was observed in cell viability at this time point within the culture conditions being compared. There was a remarkable correlation between prolonged PMN half-life and their adherence to plastic. The technique of counting all cells attached to the chamber-slide helped to assure that cells which had clumped together, or conversely, miscounted cells by not selecting representative areas of the culture chamber were not being missed. In fact, clumping of adherent cells and clustering of cells along the outside edge of the culture chamber was observed. Another advantage of manually counting the adherent cells following DifQuick staining was that differentiating between PMNs, eosinophils and contaminating monocytes was possible. Although the contaminating monocytes represented a very small percentage, typically less than 1-4%, the number of eosinophils varied between 5-20% of the total population. The inhibition of human PMN apoptosis has been investigated *in vivo* for the first 12-24 hours after the induction of an inflammatory response [200,201]. Previous data has indicated that a defined set of signals involved in inflammation promote survival of human PMNs, and that this effect is mediated by interfering with the genetic program leading to cell death. Prolongation of survival, important for the regulation of host resistance and inflammation, may represent a crucial permissive step for a defined set of cytokines and microbial products that activate gene expression and function in PMNs. It was difficult, however, to interpret these studies due to the rapid recruitment of PMNs to sites

of inflammation. In this investigation, we confirmed that the lifespan of bovine PMNs can be significantly prolonged when stimulated with cytokines and bacterial products which may be present at sites of inflammation. These observations have significant implications for the potential of PMNs to eliminate invading pathogens and regulate or propagate adaptive immune responses. Our understanding of the role played by PMNs in immune responses is far from complete, and this data challenges previous concepts regarding the brief time-frame during which PMNs were though to contribute to the inflammatory process.

CHAPTER 4

PRODUCTION OF BOVINE ADENOVIRUS TYPE-3 VECTOR EXPRESSING GREEN FLUORESCENT PROTEIN

4.1 Introduction

Bovine adenoviruses (BAdVs) belong to the *Mastadenovirus* genus of *Adenoviridae* family. Currently, the accepted 10 serotypes of BAdVs are divided into two subgroups on the basis of the differences in their biological and serological properties [202]. Serotypes 1, 2, 3, and 9 belong to subgroup I and grow relatively well in established bovine cell lines. BAdV type 3 (BAdV-3) has been extensively studied with the goal of developing it as an expression vector. Replication-defective, recombinant adenoviral vectors are widely used for *in vivo* gene transfer because of the ability of these vectors to enter many different target cells and efficiently express transgenes [203].

This laboratory has developed a recombinant bovine adenovirus vector with the gene coding green fluorescent protein (GFP) inserted in the E3 region. This vector (BAV304) was constructed such that its reporter gene can be used to trace BAdV-3 infection through transgene expression in host cells. The GFP fluoresces a bright green colour when exposed to UV light and facilitates a rapid quantification of both virus infection and transgene expression. Thus, it is possible to use flow cytometry to analyze the phenotype of cells that are infected by BAdV-3 through expression of the GFP transgene.

The purpose of this study was to use BAV304 in order to determine if the level of BAdV-3 infection in bovine PMNs would change after PMNs had passively acquired membrane and associated integral membrane protein from a BAdV-3 permissive cell line (293-cells). It was hypothesized that increased BAdV-3 infection of PMNs, detectable as increased GFP expression, would be observed following PMN passive acquisition of viral receptor protein from 293-cell membranes.

4.2 Materials and Methods

4.2.1 BAdV-3 Culture

The WBR-1 strain of BAdV-3 was cultivated in Madin-Darby bovine kidney (MDBK) and VIDO R2 cells. VIDO R2 is a transformed fetal bovine retina cell (FBRC) line expressing the E1 proteins of human adenovirus type-5. The cells were grown in Eagle's minimum essential medium supplemented with 5% fetal bovine serum.

4.2.2 Construction of Plasmid pFBAV304

The recombinant plasmid vectors were constructed by standard procedures [204] using restriction enzymes and other DNA-modifying enzymes as directed by the manufacturers. Specifically, the green fluorescent protein (GFP) gene under the control of the cytomegalovirus (CMV) immediate-early promoter and bovine growth hormone poly(A) signal was removed from the pQBI 25 plasmid (Quantum Biotechnologies) by *Bgl*II and *Dra*III digestions followed by blunt ending with T4 DNA polymerase. This fragment was first cloned into the *Srf*I site of the E3 transfer vector pBAV-301 (plasmid constructed by ligating the 7,635-bp *Kpn*I-*Ssp*I fragment from pFBAV302 to *Kpn*I-*Not*I [T4 treated]-digested plasmid PpolyIIsn14) in the same orientation as the E3 transcription

unit to generate pBAV301.gfp. A *Kpn*I-*Swa*I fragment encompassing the modified E3 region was isolated from pBAV301.gfp and recombined with *Srf*I-digested pFBAV.302 DNA [205] in *E. coli* BJ5183, creating plasmid pFBAV304.

4.2.3 Isolation of Recombinant BAdV-3

VIDO R2 cell monolayers in 60-mm-diameter dishes were transfected with 5 to 10 μg of *Pac*I-digested pFBAV304 recombinant plasmid DNA by using Lipofectin (Gibco/BRL). After incubation at 37°C, the transfected cells showing cytopathic effects were collected and freeze-thawed twice, and the recombinant viruses were plaque purified on VIDO R2 cells.

4.2.4 Western Blot Analysis of GFP

Extracts of mock- or virus-infected cells were resolved (5 μg per lane) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). Nonspecific binding sites on the membrane were blocked with 1% bovine serum-albumin before incubation of the blots with GFP protein-specific polyclonal antibodies (Clontech). The membranes were washed and exposed to anti-mouse or anti-rat immunoglobulin G conjugated to horseradish peroxidase or alkaline phosphatase and developed by using a horseradish peroxidase or alkaline phosphatase color development kit (Bio-Rad).

4.3 Results

4.3.1 Construction of Replication-Competent BAdV-3 Expressing GFP

Plasmid pFBAV304 DNA (Figure 4.1) was digested with *Pac*I and transfected into VIDO R2 cells to isolate recombinant virus BAV304. BAV304 was amplified in MDBK cells, and viral DNA was extracted from the infected cells. The viral DNA was analyzed by agarose gel electrophoresis after digestion with restriction enzyme *Bam*HI. We observed a 2.3-kb DNA fragment in the BAV304 genome (Figure 4.2A, lane 3) which was absent in the BAV3.E3d genome (Figure 4.2A, lane 2). This suggested that the BAV304 genome contained the GFP gene, a possibility confirmed by Southern blot analysis (Figure 4.2B, lane 3). A similar 2.3-kb DNA fragment was also observed in the pFBAV304 genome (Figure 4.2A, lane 4) but not in pFBAV302 (lane 5). This suggests that pFBAV304 contained a GFP gene, a possibility confirmed by Southern blot analysis (Figure 4.2B, lane 5).

To examine the expression of GFP, recombinant BAV304-infected cell lysates were analyzed by Western blotting using GFP-specific polyclonal antibodies (Clontech). The anti-GFP antibodies identified a band of 28 kDa in recombinant BAV304-infected cells (Figure 4.3, lanes 1 to 3). No such band was observed in mock (lane 4)- or wild-type BAdV-3 (lane 5)-infected cells. In BAV304-infected cells, GFP was detected from 12 to 36 h postinfection (lanes 1 to 3).

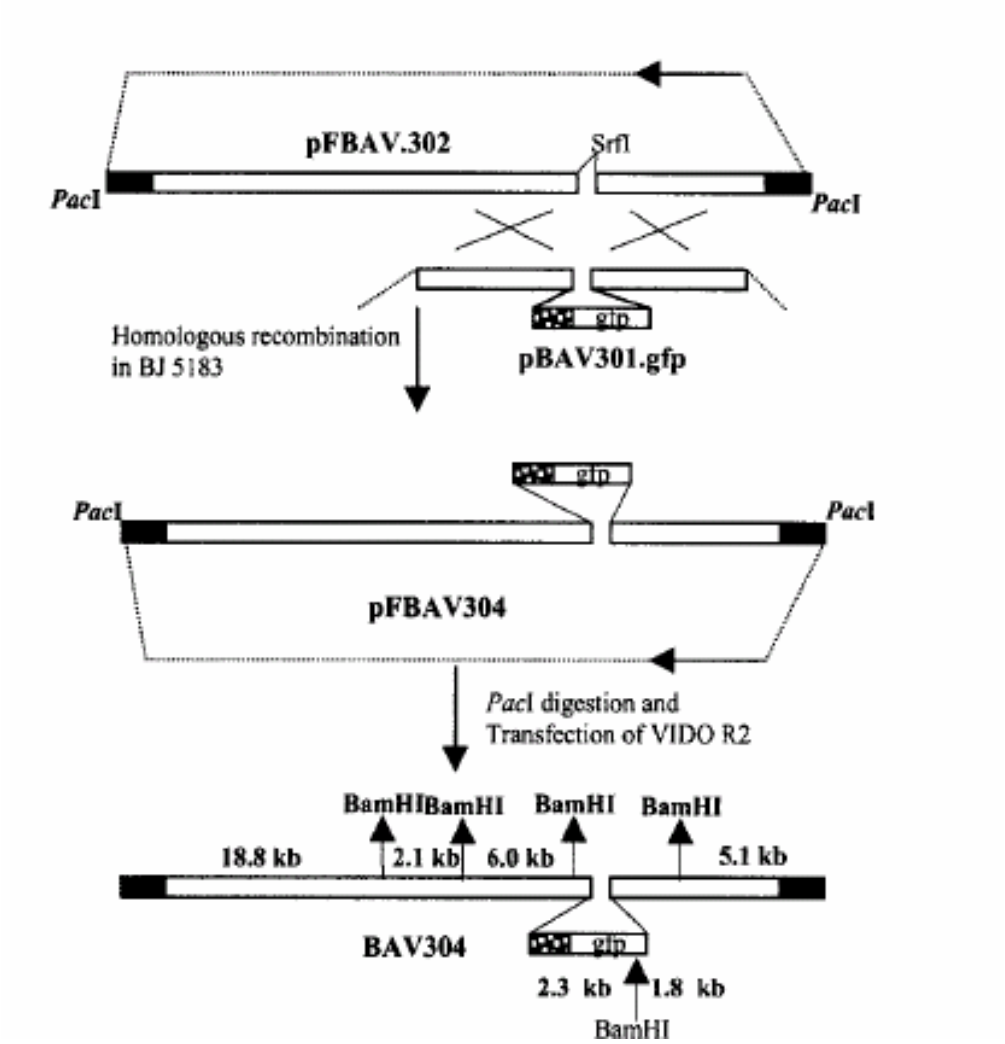


Figure 4.1. Strategy used for the generation of recombinant BAV304 vector. The GFP plasmid was constructed as described in the text. Origins of DNA sequences: plasmid DNA, thin line; BAdV-3 genomic DNA, hollow box; inverted terminal repeats, filled box. The plasmid maps are not drawn to scale.

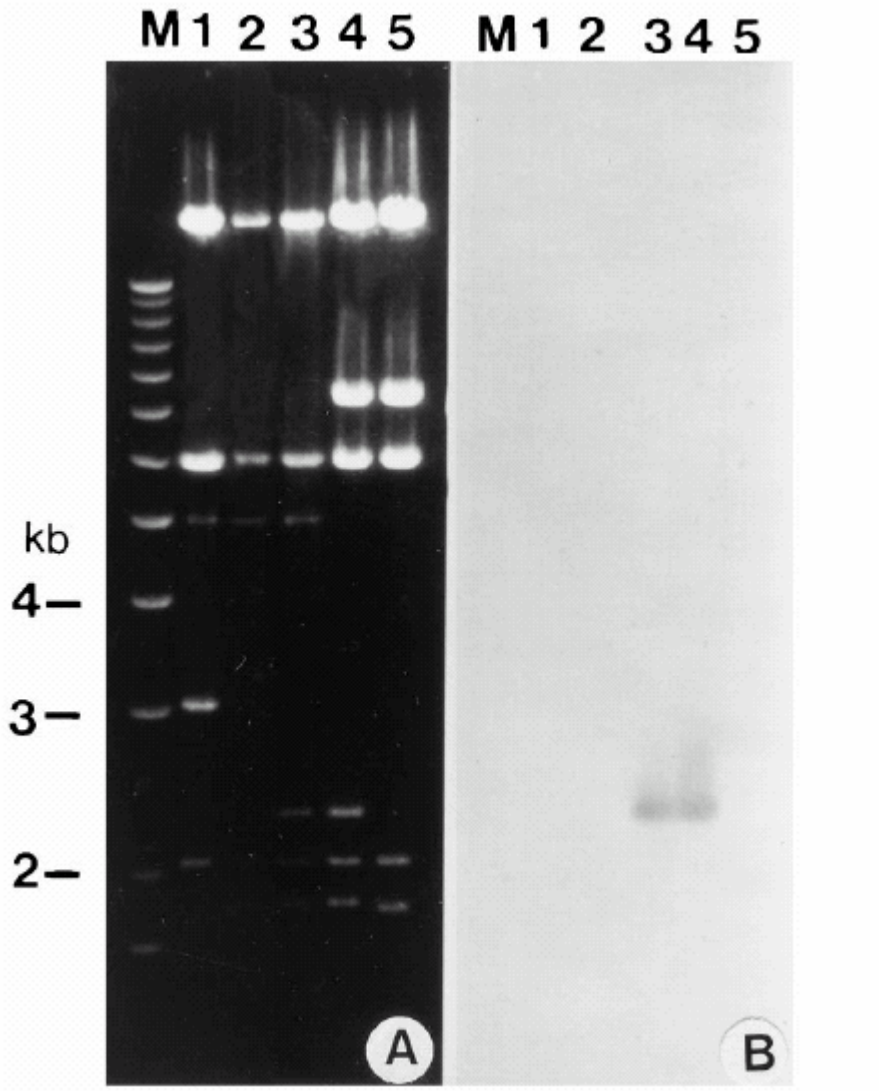


Figure 4.2. Analysis of recombinant BAV304 genome. (A) The DNAs were extracted from BAdV-3 (lane 1), BAV3.E3d (lane 2), BAV304 (lane 3), pFBAV304 (lane 4), and pFBAV302 (lane 5) digested with *Bam*HI and analyzed by ethidium bromide staining of an agarose gel. (B) The fragments shown in panel A were transferred to a Nytran membrane and probed with a α-^{32}P-labeled GFP gene. Lane M, 1 Kb Plus DNA Ladder (Gibco/BRL) used for sizing the DNA fragments.

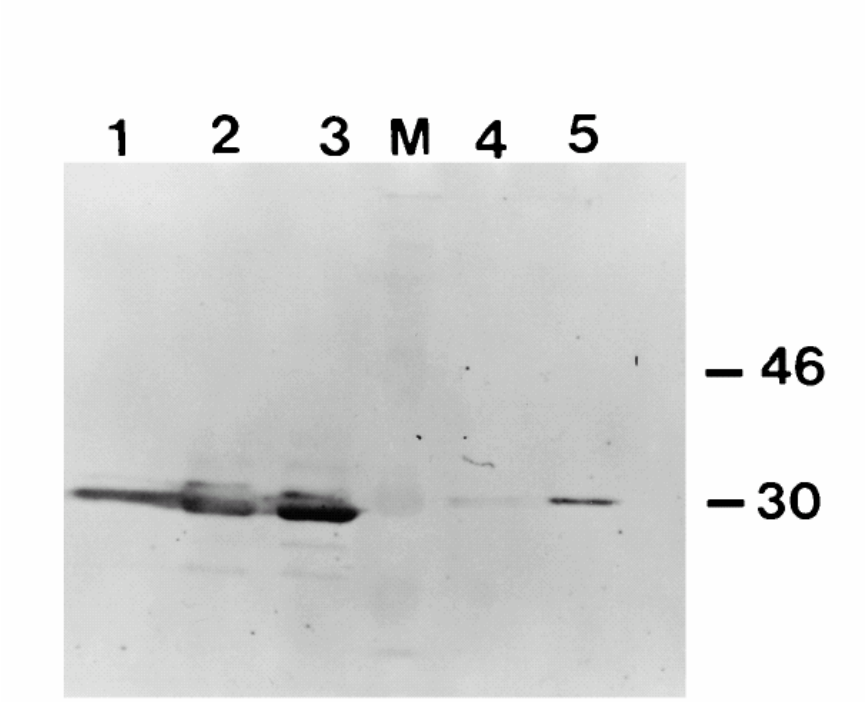


Figure 4.3. Western blot analysis of GFP expression. Proteins from mock (lane 4)-infected, BAdV-3-infected (lane 5), or BAV304-infected MDBK cells harvested at 12 (lane 1), 24 (lane 2), and 36 (lane 3) h postinfection were separated by SDS-PAGE (10% gel) under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blots by GFP-specific polyclonal antiserum. Molecular masses (lane M) are indicated in kilodaltons.

4.4 Discussion

The BAV304 vectors were produced in a bovine kidney cell line (MDBK) and purified using a standard adenovirus purification technique. Purification was deemed necessary in order to eliminate the possibility that soluble GFP protein might be phagocytosed by PMNs. Soluble protein could give a false positive reading for transgene expression in PMNs. Infectious BAdV-3 titre was markedly reduced during the purification process. Therefore, establishing an accurate virus titration method was of major importance. This was achieved by counting the number of fluorescing plaques that BAV304 formed on an MDBK monolayer. This is an accurate technique since each plaque is distinct at high viral dilutions, and plaques can be counted as early as 2-3 days after infection. An accurate titre, or at least a reliably consistent titration method is important for establishing the multiplicity of infection (MOI) number. For this reason, all the vectors used throughout our experiments were first produced and then a standardized pool of virus was used throughout subsequent experiments.

As GFP expression in cells is easily detected using flow cytometry, the BAV304 construct was an effective tool for determining the tropism of BAdV-3 for specific cell populations. Our intended use for this vector was to determine whether PMNs were able to passively acquire a functional BAdV-3 receptor.

CHAPTER 5

BOVINE POLYMORPHONUCLEAR CELLS PASSIVELY ACQUIRE MEMBRANES AND INTEGRAL MEMBRANE PROTEINS FROM APOPTOTIC AND NECROTIC CELLS

5.1 Abstract

Leukocytes can acquire membrane fragments and integral membrane proteins from dead and dying cells, and in some cases, from live cells. While investigating the possibility that bovine polymorphonuclear cells (PMNs) might present antigen, co-culture assays confirmed that integral membrane proteins were rapidly and efficiently transferred to bovine PMNs from a variety of apoptotic and necrotic cells. Specifically, rapid acquisition of such proteins as MHC Class II and CD3 was shown from a variety of syngeneic, allogeneic and xenogeneic cell types. Such acquisition occurred within 40 minutes of PMN co-culture with isolated peripheral blood mononuclear cells (PBMCs) and was not inhibited at 4^{o}C. The transfer of murine MHC II molecules precluded the possibility of endogenous expression. We also demonstrated the transfer of fluorescently labelled membrane lipids along with integral membrane proteins. Collectively, these observations support the hypothesis that membrane protein transfer was mediated by the fusion of membrane fragments or microvesicles with the PMN plasma membrane and not by phagocytosis of cell fragments. These observations indicate that phenotypic studies of PMNs must consider circumstances whereby PMNs may passively acquire membrane proteins due to the impressive capacity of PMNs to acquire membrane lipids and a variety of integral membrane proteins from dead or dying cells.

5.2 Introduction

Historically, PMNs have been regarded as terminally differentiated effector cells. The principle role of the PMN was thought to be phagocytosis with an impressive assortment of preformed antibacterial systems and soluble mediators that provide a relatively rapid defence against pathogens and tissue damage. However, the role of PMNs within the immune system may be much broader. For example, PMN derived cationic peptides can influence cell trafficking and dendritic cell activation [206] and PMNs have also been shown to express an impressive assortment of proteins and cytokines such as CR1, CR3, FcR, IFN-α, IL-1, IL-3, IL-8 and TNF-α [92]. These observations provide further evidence that innate and adaptive immune responses are closely integrated.

Until recently, there was no evidence to suggest that PMNs could play a role in the induction of an adaptive immune response [92], however, there are several reports that PMNs from a variety of species can express MHC Class II proteins and co-stimulatory molecules (CD80 and CD86) [80,88,207,208]. Co-expression of MHC Class II and co-stimulatory molecules would potentially endow PMNs with the capacity to influence the adaptive immune system through antigen presentation. Previous investigations with mouse, human and goat PMNs defined conditions where MHC II expression was observed following stimulation with IFN-γ, GM-CSF or IL-3 [78,79,81,82], or in human patients with Wegener's Granulomatosis [89,209]. In some cases, PMN expression of MHC II was reported as constitutive [77,80]. Human PMNs were also shown to function as accessory cells for primed T-cell activation with protein antigens and super-antigens [62,88,89,210]. Earlier observations, that recombinant bovine adenovirus vaccine vectors had a tropism for PMNs, triggered an interest in

determining the role of PMNs in the induction of a specific immune response. Since bovine adenoviral vectors can stimulate strong immune reactions [211], the hypothesis that bovine PMNs might function as antigen presenting cells (APCs) was tested, thereby providing a link between the innate and adaptive branches of the immune system. As there was a growing body of literature to suggest that PMNs from other species could assume such a role, we began by investigating the expression of MHC Class II, a key protein involved in antigen presentation, on bovine PMNs. It was observed that bovine PMNs expressed detectable levels of MHC Class II on their surface only when co-cultured with PBMCs. This observation suggested that PMNs may either up-regulate endogenous MHC II expression or passively acquire MHC II protein from other leukocytes.

Recent observations suggest that cell membrane fragments are readily exchanged between certain immune cells [134,138-141]. Although the consequences of this event are not fully understood, several interesting theories are emerging to explain its significance. 'Cross-presentation', for example, is a term coined for the circumstance where a dendritic cell (DC) can acquire antigen from another cell and present this antigen on endogenous MHC Class I. What is especially unique about this process is that DCs are able to sample both extracellular membranes and intracellular proteins from live cells [134]. Other cell types, such as macrophages, can only acquire cytoplasmic membranes from dead or dying cells [134]. T-cells have also been shown to acquire membrane proteins from antigen presenting cells in an activation dependent manner [138,140]. In particular, T-cells can acquire MHC Class II proteins and co-stimulatory molecules, which support antigen presentation to other primed T-cells [138,140]. Furthermore, some B-cell lines are capable of a continuous

uptake of autologous cell membrane and proteins through an immunological synapse [147].

The purpose of this study was to investigate if MHC II detected on the surface of bovine PMNs was the result of passive acquisition or due to endogenous synthesis of MHC II protein. The data presented demonstrate that bovine PMNs have a remarkable capacity to passively acquire both plasma membrane lipids and a variety of integral membrane proteins from several types of dead or dying cells.

5.3 Materials and Methods

5.3.1 Culture Media and Reagents

All cell cultures were maintained in AIM-V Serum Free Lymphocyte Medium (Gibco BRL, Burlington, ON) containing 20% (v/v) heat inactivated fetal bovine serum (FBS; Gibco BRL) and 50 μM 2-mercaptoethanol (2-ME; Biorad; Mississauga, ON). Recombinant human IL-2 was purchased from R&D Systems Inc. (Minneapolis, MN), recombinant bovine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) was from Genentech Inc. (San Francisco, CA), lipopolysaccharide (LPS) was from Sigma-Aldrich (Oakville, ON) and recombinant bovine interferon-gamma (IFN-γ) from Ciba-Geigy (Basel, Switzerland).

5.3.2 Purification of Cells

Blood was collected from the jugular vein of male or female Holstein cattle (age > 12 months) using 0.3% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). Whole blood was centrifuged at 1400 x g, without braking, for 20 minutes to separate the buffy coat layer, which was removed at the interface between the red blood cell pellet and the

plasma, diluted 1:1 with calcium and magnesium-free phosphate buffered saline (PBSA; 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.3) and layered onto Ficoll-Hypaque (Pharmacia LKB, Biotechnology, Uppsala, Sweden) and centrifuged at 1400 x g (without braking) for 20 minutes, to obtain a mononuclear cell population. After removal of plasma and buffy coat, the top half of the remaining red blood cell/PMN (RBC/PMN) fraction was also discarded. RBCs were lysed by adding 5-10ml of the RBC/PMN fraction to 40ml of a lysis solution (distilled water with 0.17M NH_4Cl, 10mM $KHCO_3$, and 0.11mM EDTA; pH 7.3) followed by three washes with PBSA. Cells prepared by this method were consistently >98% viable as determined by trypan blue dye exclusion and contained less than 4% mononuclear cells. No attempt was made to differentiate between PMN and eosinophilic granulocytes, but the latter were usually present in low numbers as determined by cell counts using DifQuick (Baxter Scientific, Miami, FL) stained cytospins.

To obtain an enriched population of B-cells, PBMCs were collected, resuspended in PBSA at 4×10^7 cells/ml, and incubated with 1ml of both MM1A (CD3; VMRD, Pullman, WA) and DH59B (CD179a; VMRD) sodium azide-free monoclonal antibodies (mAb) at 5μg/ml for 30 min on ice. Cells were then incubated with goat anti-mouse IgG-coated magnetic beads (Dynabeads® M-450, Dynal, Great Neck, NY) at a bead to target cell ratio of 5:1. Bead-bound cells were removed with a magnet as described by the manufacturer and the remaining cells were washed in PBSA and resuspended in AIM-V medium at $1x10^7$ cells/ml.

Clone 2 B-cells (a sheep B-cell line) were cultured as a source of B-cells that undergo low level apoptosis in culture as previously described [212].

An enriched T-cell (CD3 cells) population was obtained by culturing 5×10^6 PBMCs in 5ml of Aim-V medium with 5% v/v FBS, 4μg/ml Concanavalin A (Sigma-Aldrich) and 4ng/ml of human IL-2. Cells were passaged and the media was replaced every 2-3 days. This culture system produced an activated T-cell population (>99% $CD3^+$) which expressed MHC Class II (~ 50% MHC II^+).

Activated PMNs were collected from the mammary gland of heifers following injection into each teat canal of 5ml of minimal essential medium (MEM) containing 1μg/ml LPS (Sigma-Aldrich). PMNs were collected approximately 16 hours after LPS injection by infusing each teat with 10ml of MEM, massaging the mammary gland and then expressing the liquid from the teat canal into a sterile bottle. This procedure was repeated with 5ml of MEM. Cytospins stained with DifQuick (Baxter Scientific, Miami, FL) were examined with a microscope and PMN purity was consistently 95-98%. Approximately 4 days after stimulation of the mammary gland, large numbers of macrophages were recruited to the mammary gland and were collected in the same way as for PMN collection to generate an enriched macrophage population [213].

Transwell separated co-cultures were performed using a 0.4μm transwell plate (Corning Costar, Cambridge, MA) in order to eliminate direct cell-cell contact between the PMN population (cultured in the bottom well) and the cell population contained in the upper chamber. The transwell system was selected to allow for the exchange of soluble products, while eliminating access of intact cells or larger cell fragments to the bottom well. All cell cultures were incubated at 37^oC in 5% CO_2 and all co-cultures were carried out in 6-well plates with $1\text{-}3x10^6$ PMNs/well at a 1:3 ratio (PMN: Other cell) with 6ml of AIM-V medium (20% FBS) unless otherwise specified.

5.3.3 Biotin and fluorescence labelling

Cells were labelled with PKH 26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) according to the manufacturer's instructions. Similarly, according to the manufacturer's instructions, viable cells were labelled with EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL). Biotinylation of surface proteins was confirmed by flow cytometric analysis of streptavidin-fluorescein isothiocyanate (Southern Biotechnology Birmingham, AL) binding.

5.3.4 Induction of apoptosis or necrosis

Cell lysis was performed by three cycles of snap-freezing in liquid nitrogen and thawing in a 37^{o}C water bath (freeze/thaw, 1x10^{7}cells/ml). Cells were then sonicated for 20 seconds using a microtip vibrating at 20 kHz and amplitude of 40%.(VibraCell ™ Sonicator, Betatex Inc.). To induce apoptosis, cells were gamma-irradiated with approximately 11000rads. To confirm the induction of apoptosis the TUNEL assay (Flow TACS ™ *In Situ* Apoptosis Detection Kit, R&D Systems, Minneapolis, MN) was used.

5.3.5 Membrane extraction

Clone 2 ovine B-cells or bovine B-cells were washed two times in PBSA and 8x10^{7} cells were resuspended in 2ml of homogenisation buffer (20mM Tris HCl pH 7.5, 10mM NaCl, 0.1mM $MgCl_2$, 10mM PMSF). Cells were sonicated for 20s, underlaid with 5ml of 40% sucrose (in homogenisation buffer), and centrifuged at 75,000 x g for 1h at 4^{o}C. The white floccular material at the interface between the buffer and sucrose was

collected, diluted in PBSA and pelleted at 210,000 x g for 1hr at 4^{o}C. The pellet was resuspended in PBSA and stored at –70^{o}C.

5.3.6 Flow cytometry

Monoclonal antibodies (mAb) specific for bovine CD3 (Clone MM1A), CD14 (Clone MM61A), CD21 (Clone Baq15A), CD179a (Clone DH59B) and the DR region of bovine MHC II (Clone TH14B) were purchased from VMRD (Pullman, WA). Rat-anti-mouse I-A/I-E was purchased from Becton-Dickinson (Missisauga, ON) and FITC labelled goat-anti-rat IgG from Caltag Laboratories (Burlington, CA). Fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated, isotype-specific goat anti-mouse immunoglobulin (Ig) antibodies were purchased from Southern Biotechnology. The level of specific mAb binding was quantified by subtracting cells that reacted with an equivalent concentration of an isotype-matched, irrelevant mAb (Cedar Lane Laboratories Ltd., Ontario). Cells were fixed in 2% paraformaldehyde in PBS (same as PBSA with pH of 6.7) and stored in the dark at 4^{o}C until data acquisition and analysis was performed with a FACScan flow cytometer using the Cell Quest program (Becton Dickinson, Mountain View, CA).

5.3.7 Statistical Analysis

All of the results presented are non-parametric in their distribution. In the cases where results are paired, the Wilcoxon matched pairs t-test was used. When individual data sets were not paired, then the Mann-Whitney t-test was used.

5.4 Results

5.4.1 Detection of MHC Class II on freshly isolated bovine PMNs

Initial investigations explored the possibility that bovine PMNs were able, as had been observed in other species, to endogenously express MHC class II proteins. Such expression was reported to be either inducible or constitutive. It is interesting to note, however, that a previous report suggested that expression of MHC Class I and II proteins was significantly reduced following the use of centrifugation to isolate caprine PMNs [80]. Consequently, we decided to first determine if the cell isolation procedure affected MHC II expression on bovine PMNs. Fresh whole blood was lysed to remove erythrocytes and the PMN population was analysed for MHC class II expression using flow cytometry. There was marked animal to animal variation in the percentage of PMNs with a detectable level of MHC II (Range = 7-36%), but PMNs from all animals had detectable surface MHC II (Figure 5.1A). We then investigated whether bovine PMNs expressed detectable levels of MHC II following isolation using centrifugation speeds of 1400 x g to first separate the mononuclear cells (buffy coat) from PMNs. Bovine PMNs isolated following centrifugation of whole blood had little detectable surface MHC II protein (Range = 0.1-2.5%). To determine if the time between blood collection and cell isolation might affect the level of MHC II expression, we incubated whole blood for 24 hours prior to PMN isolation. PMNs incubated for 24 h in whole blood had significant levels of detectable MHC II following isolation by either centrifugation (Range = 3-36%) or whole blood lysis (Range = 11-56%) (Figure 5.1A). In addition, MHC Class II (Range = 0-22%) was detected on PMN collected directly from the bovine mammary gland without any post-collection manipulation (Figure 5.1A). In all experiments, we did not observe the acquisition of MHC II by a specific subset of PMNs but rather a general acquisition

of protein by all PMNs as evidenced by a population shift in the FACS profile (Figure 5.1B). These observations suggested that both the interval between blood collection and bovine PMN isolation, as well as the method used for PMN isolation could significantly alter the level of MHC II detected on the cell surface. The mammary gland data also confirmed that MHC II may be present on the surface of PMNs *in vivo*.

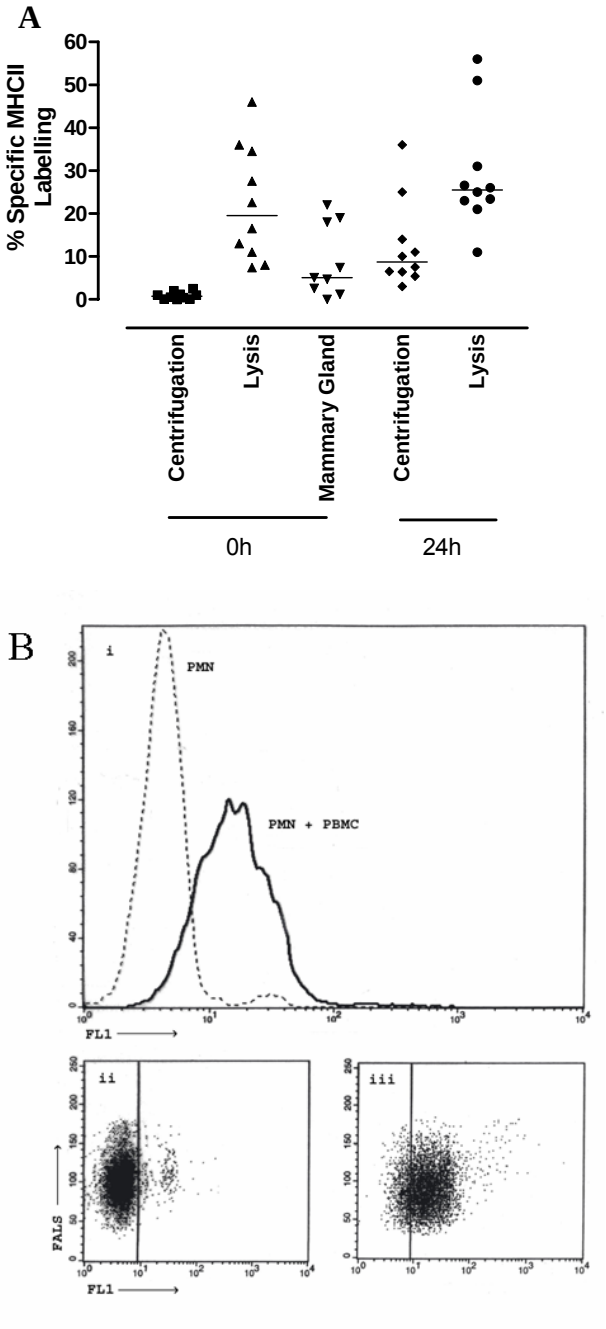


Figure 5.1. PMN isolation method alters detection of MHC class II protein on the surface of bovine PMNs. (A) Three isolation techniques were compared: Centrifugation (n=10) - purified PMNs were isolated following centrifugation to separate mononuclear cells; Lysis (n=10) - whole blood lysis to remove erythrocytes and leave all leukocytes; and isolation of PMNs from the mammary gland following LPS injection to induce inflammation and recruitment of an enriched PMN population (n=6). Data presented are values for individual animals (medians indicated in each group) with blood samples from the same animals used to compare each isolation method. The centrifugation and lysis techniques were also compared at 0h and 24h after blood collection. There was a significant difference in % MHC II labelling for the following: 1) centrifugation vs lysis at 0h and 24h ($P<.001$ and .004 respectively), 2) centrifugation at 0h vs 24h ($P=.002$), 3) mammary gland vs lysis at 0h ($P=.02$), 4) mammary gland vs centrifugation at 0h ($P=.008$). There was no significance ($P>.05$) between the two lysis groups. (B) A representative FACS histogram (i) and dot plot (ii,iii) of MHC II stained bovine PMNs following a 24h co-culture with irradiated PBMCs (i - solid line; profile in iii) or PMN alone (i - dotted line; profile in ii).

5.4.2 Induction of MHC II Expression

Previous investigators reported that MHC II expression could be induced when human PMNs were cultured with GM-CSF or IFN-γ [78,79,81,82]. Following incubation of purified bovine PMNs with recombinant bovine GM-CSF (1ng/ml), IFN-γ (10 or 100 ng/ml), LPS (0.5ug/ml) or a combination of all three, no MHC II expression was detected (Figure 5.2A). Only when PMNs were co-cultured in direct contact with PBMCs was surface MHC Class II protein detected on PMNs by flow cytometry (Figure 5.2B).

It has also been reported that a soluble T-cell factor can induce MHC II expression on human PMNs [88]. To determine if a soluble T-cell factor might induce MHC II expression on bovine PMNs, purified PMNs were co-cultured for 24hr with a 0.4μm transwell membrane separating PBMCs or ConA/IL-2 activated T-cell populations. The transwell membrane facilitates an exchange of secreted soluble factors but prevents direct cell-cell contact. PMNs co-cultured with PBMCs or activated T-cells by this method showed no detectable MHC II expression (Figure 5.2B). These observations supported the conclusion that direct cell-cell contact was required for PMNs to express MHC II molecules on their surface.

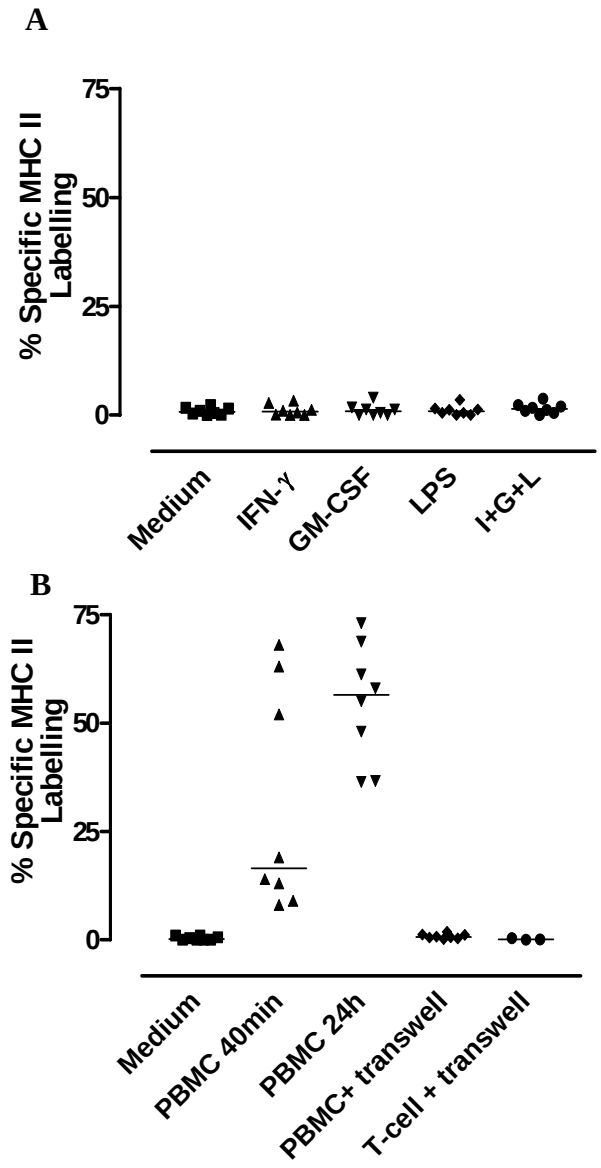


Figure 5.2. PMN stimulation does not alter surface expression of MHC class II protein. (A) Following 24h stimulation of PMNs with medium alone, various cytokines, or a combination of all three (I+G+L). Cytokine concentrations were as follows: IFN-γ - 100ng/ml, GM-CSF - 1ng/ml, LPS - 0.5μg/ml. Data presented are values for individual animals (medians indicated in each group) with PMNs from each animal (n=8) used to compare all culture condition There were no significant differences in any group when compared to the media control ($p>.05$). (B) PMNs were also co-cultured for 40 minutes or 24h in direct contact with PBMCs (PBMC 40min, PBMC 24h). Both of these groups were significantly different from the media control ($P<.008$). PMNs were also co-cultured for 24h with PBMCs (PBMC+transwell) or activated T-cells (T-cell+transwell) and separated by a 0.4μm transwell membrane. Transwell cultures were not significantly different from the media control. Data presented are values for individual animals with the median value indicated for each group.

5.4.3 Passive Acquisition of MHC Class II

Since under defined isolation proceedures no evidence that purified bovine PMNs could be induced to express endogenous MHC II was observed, the hypothesis that PMNs might passively acquire MHC II was tested. To test this hypothesis, the kinetics of MHC II expression on PMNs following co-culture with PBMCs was first examined. Throughout the co-culture experiments, we used a donor cell:PMN ratio of 3:1 as an approximation of the ratio of PBMC:PMN found in bovine blood under normal physiological conditions [214]. Detectable levels of MHC Class II DR molecules on PMNs within 40 minutes of co-culture at 4^{o}C was observed (Figure 5.2B). A parallel experiment was performed at 37 ^{o}C and no significant difference in MHC II acquisition was observed (data not shown). This experiment demonstrated that MHC II proteins were rapidly acquired by PMNs when exposed to PBMCs and the acquisition of MHC Class II molecules at 4^{o}C further supported the hypothesis that MHC II molecules were passively acquired by bovine PMNs.

The final experimental approach used to confirm that bovine PMNs had passively acquired MHC II molecules was to analyse the transfer of murine MHC class II proteins from murine splenocytes to bovine PMNs. The monoclonal antibody specific for murine MHC II (I-A^{d}/I-E^{d}) did not cross-react with bovine MHC II (Figure 5.3). Therefore, we were able to demonstrate that following PMN co-culture with murine splenocytes the MHC II detected on the surface of bovine PMNs was of mouse origin. This provided unequivocal evidence for the passive transfer of MHC II (Figure 5.3).

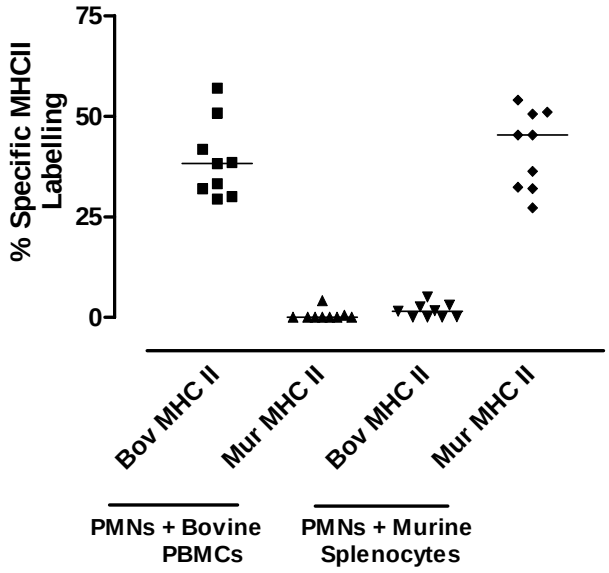


Figure 5.3. Passive transfer of murine MHC class II protein to bovine PMNs following a 24h co-culture with murine splenocytes. The antibody used to detect species-specific MHC II and co-culture conditions are indicated beneath the x-axis. Comparison within each co-culture group revealed a significant difference ($P<.004$). The data presented are values for individual animals (n = 9) and median value is indicated for each group.

5.4.4 Characterization of MHC Class II Donor Cell

Investigation of which leukocytes could donate MHC Class II molecules to PMNs was examined. Enriched populations of bovine T-cells (activated to induce MHC II expression), B-cells, and monocytes were prepared and each population was co-cultured with PMNs. These experiments revealed that only B-cells efficiently transferred MHC Class II protein to the surface of PMNs (Figure 5.4B). Most B-cells (>60%), however, die soon after being isolated and cultured (Figure 5.4A), therefore it was hypothesized that apoptotic cell fragments might be the source of MHC II molecules passively acquired by PMNs. To further investigate the mechanism by which integral membrane proteins are transferred to PMNs it was determined if the physiological state of the donor cell influenced this process. Passive acquisition of MHC Class II by bovine PMNs was analyzed following co-culture with viable or necrotic cells and isolated cell membranes (Figure 5.4B). To confirm that apoptosis was required for the efficient transfer of integral membrane molecules, co-cultures of bovine PMNs with bovine B-cells and a sheep B-cell line (clone 2 cells) which has a low level of apoptosis in culture (data not shown) were compared. There was no detectable transfer of MHC II protein to bovine PMNs following co-culture with sheep B-cells unless the B-cells were physically disrupted by repeated freeze/thaw and sonication. Following this B cell treatment, PMN acquisition of ovine MHC Class II was equivalent to that observed previously with bovine B-cells (Figure 5.4A**).**

Finally, the possibility that the passive acquisition of membrane proteins by bovine PMNs might represent a phenomenon which was not restricted to MHC Class II proteins was addressed. The co-culture of purified bovine T-cells and PMNs

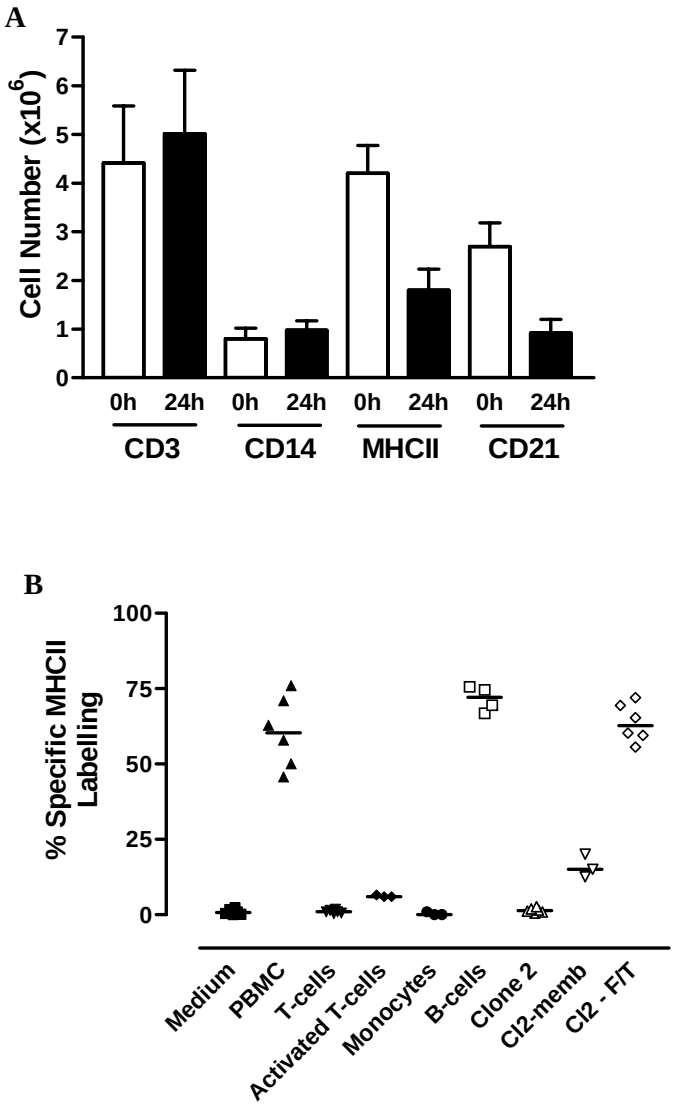


Figure 5.4. Identification of MHC II donor cell phenotype. (A) PBMCs were labelled with anti-MHC II, anti-CD3 (T-cells), anti-CD14 (monocytes) and anti-CD21 (B-cells) specific monoclonal antibodies to monitor changes in cell number during a 24h co-culture with PMNs. A significant reduction in both MHC II^+ ($P<.005$) and $CD21^+$ ($P<.006$) cells was observed. (B) PMNs were co-cultured for 24h with MACS purified bovine cell populations (T-cells = $CD3^+$; monocytes = $CD14^+$; B-cells = [PBMCs – ($CD3^+$ + $C14^+$)]). Viable clone 2 sheep B-cells (Clone 2), isolated Clone 2 membranes (Cl2-memb), and freeze-thaw and lysed clone 2 B-cells (Cl2-F/T) were also co-cultured with bovine PMNs for 24h. Data presented are values for individual animals with median values indicated for each group. Compared with the media control, significant differences were observed following PMN co-culture with PBMCs ($P<.003$), activated T-cells ($P<.03$), B-cells ($P<.01$), clone 2 membranes ($P<.03$), and lysed clone 2 cells ($P<.003$).

resulted in the transfer of detectable CD3 protein on approximately 17.0 % of PMNs. Furthermore, gamma-irradiation of bovine T cells to induce apoptosis (confirmed by TUNEL assay) resulted in a significant increase in the percentage of PMNs with detectable CD3 on their surface (Figure 5.5). The detection of CD3 on the surface of PMNs provided further evidence for the passive acquisition of integral membrane proteins and suggested that a variety of molecules could be acquired from apoptotic cells.

5.4.5 Passive Acquisition of Membranes and Integral Membrane Proteins

Passive acquisition of MHC II and CD3 molecules suggested that PMN acquisition of integral membrane proteins might reflect a more general process. Two different labelling techniques were then used to determine if PMNs passively acquired both membrane lipids and a broad range of integral membrane proteins. Bovine PBMCs were labelled with either the lipophilic PKH-26 dye or surface proteins were biotinylated before the PBMCs were co-cultured with PMNs. The transfer of these two labels was then monitored using flow-cytometry (Figure 5.6) and analyses supported the conclusion that PMNs passively acquired both plasma membrane lipids and integral membrane proteins.

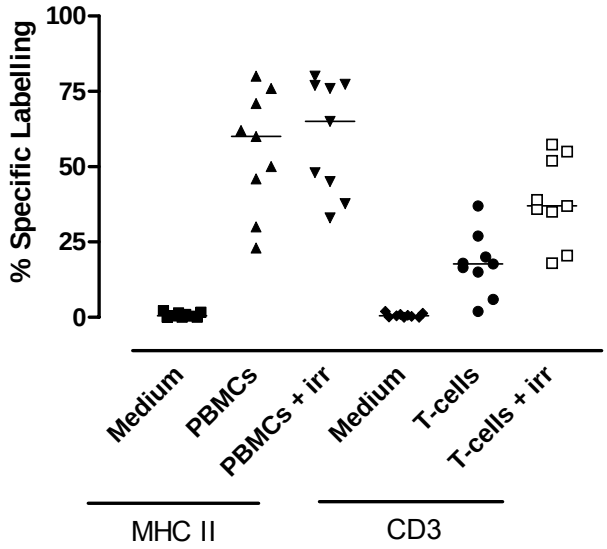


Figure 5.5. Bovine PMNs passively acquire both CD3 and MHC II molecules from bovine T cells. PMNs were co-cultured for 24h with either PBMC, T-cells or gamma-irradiated PBMC and T-cells (PBMCs + irr; T-cell + irr) and then stained with anti-MHC II or anti-CD3 monoclonal antibodies as indicated. Data presented are values for individual animals (n = 9) and group medians are indicated. All co-cultures were significantly different from their appropriate media control (P=.004). There was also a significant difference in the percentage $CD3^+$ PMNs following co-culture with non-irradiated versus gamma-irradiated T-cells (P<.008).

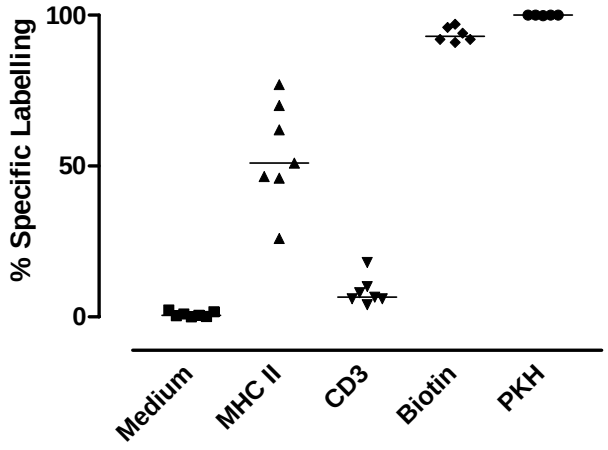


Figure 5.6. Transfer of integral membrane proteins and plasma membrane lipids from PBMCs to PMNs. Flow cytometric detection of MHC II, CD3, Biotin, or PKH molecules on bovine PMNs following a 24h co-culture with unlabelled PBMCs, biotin labelled PBMCs (Biotin) or PKH-26 (PKH) labelled PBMCs, respectively. Data presented are values for individual animals (n=7) and the median value is indicated for each group. There was a significant transfer of MHC II ($P<.001$), CD3 ($P<.001$), biotin/SA-FITC ($P<.002$), and PKH-26 ($P<.03$) when compared to the media control.

5.5 Discussion

This study demonstrates for the first time that interactions between bovine PMNs and either dead or dying cells can lead to the passive acquisition of plasma membrane lipids and a variety of integral membrane proteins. The present observations, however, contradict previous reports that human, murine and caprine PMNs can endogenously express MHC Class II molecules. Although the possibility that PMNs from other species can express endogenous MHC class II and can, therefore, act as accessory cells cannot be discounted, the *in vitro* data consistently supported the conclusion that bovine PMNs did not endogenously express MHC Class II (Figure 5.2). The presence of detectable MHC II molecules on the surface of bovine PMNs isolated directly from an inflamed mammary gland was, however, observed (Figure 5.1). These observations are consistent with previous reports that both human [9-12] and murine [13] PMNs may express MHC II *in vivo*. Thus, the possibility that physiological conditions may exist during which bovine PMNs express endogenous MHC II cannot be discounted but our *in vitro* data consistently supported the conclusion that bovine PMNs have an impressive capacity to passively acquire a broad range of integral membrane proteins during cell isolation and culture. It might therefore, be prudent to re-examine previous reports regarding PMN expression of MHC II *in vitro* to determine if an opportunity existed for the passive acquisition of membrane proteins.

A previous report that human PMNs expressed MHC II molecules did not provide sufficient technical detail to determine if passive transfer of MHC II was a possibility during cell isolation or culture [81]. Culture conditions used in this study, however, may have supported the increased expression of MHC II on contaminating

mononuclear cells. Thus, there was no assurance that MHC Class II detected on PMNs wasn't passively acquired from other contaminating cells. Radsak *et. al.* [88] used RT-PCR to confirm endogenous MHC Class II expression in PMNs, but the presence of even low numbers of either monocytes or DC could have affected PCR results. This group also suggested that a soluble T-cell factor was responsible for the induction of MHC II expression on human PMNs. In contrast, bovine PMNs co-cultured in the presence of either activated T-cell or PBMCs but separated by a 0.4um transwell membrane did not show detectable MHC II expression (Figure 5.2). Thus, the present observations consistently supported the conclusion that bovine PMNs had passively acquired MHC II molecules and studies with recombinant cytokines were consistent with a previous report that IFN-γ could not induce MHC II expression on caprine PMNs, another ruminant species [80].

Of particular importance was the rapid acquisition of MHC II molecules by bovine PMNs within 40 minutes and that this acquisition occurred with equal efficiency at 4^{o} and 37 ^{o}C. Mammalian cell transcription and translation are markedly decreased at 4^{o}C [215] and this limits metabolically active processes. Reduced membrane fluidity at 4^{o}C further restricts the potential for membrane fusion and phagocytosis. Thus, the rapid acquisition of surface MHC II protein at 4^{o}C is not consistent with phagocytosis but is more consistent with a direct attachment of membrane fragments to the PMN surface. A proposed model for such transfer of membrane lipids and integral membrane protein is

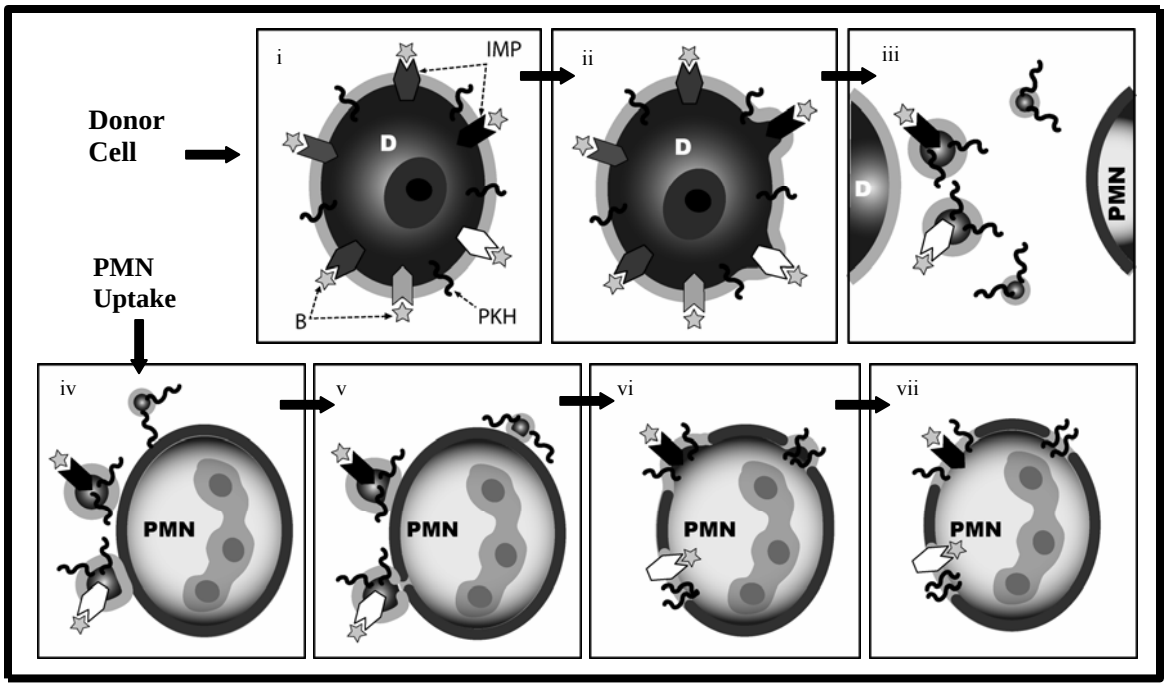


Figure 5.7. Microparticle model for the passive transfer of membrane lipids and integral membrane proteins to bovine PMNs. After the induction of apoptosis or necrosis, donor cells (D) release microparticles which can contain integral membrane proteins (IMP), or lipophilic PKH dye (PKH). These microparticles adhere to and fuse with PMN plasma membranes. IMPs inserted in the proper orientation in PMNs can then be detected using biotin (B).

shown in Figure 5.7. Cells undergoing apoptosis shed membrane fragments as small heterogeneously sized microvesicles which can contain integral membrane proteins [131]. These microvesicles exhibit increased adherent properties [216] and we hypothesize that they attach directly to the PMN surface and can then become incorporated into the PMN membrane.

The model proposed for PMN passive acquisition of integral membrane proteins is consistent with several critical observations. It was observed, as previously reported for caprine PMNs [4] that the method of PMN isolation can alter apparent MHC II expression (Figure 5.1A). The absence of detectable MHC II on PMNs following centrifugation would be consistent with the removal of loosely attached microvesicles or membrane fragments by centrifugal forces. The observation that centrifugation did not reduce surface MHC II on PMNs following incubation of blood at 37 ^{o}C for 24h may be consistent with either the phagocytic uptake of donor cell fragments by PMNs or direct fusion of microparticles with the PMN plasma membrane. If phagocytosis was involved in the passive acquisition of integral membrane proteins then a mechanism would be required for the efficient recycling of proteins from the phagolysosome to the cell surface. The observation that a variety of integral membrane proteins, such as CD3 (Figure 5.5) and biotinylated proteins (Figure 5.6), were also efficiently transferred to the PMN plasma membrane implies that a large number of proteins were recycled from the phagolysosome. MHC II molecules, when loaded with peptide, can be recycled to the cell surface but a similar process has not been reported for other integral membrane proteins.

The observation that PKH, a lipophilic fluorescent dye, was transferred from donor cells to PMNs (Figure 5.6) provided evidence that donor cell membranes were

transferred with integral membrane proteins. This observation is consistent with the hypothesis that membrane proteins were transferred to PMNs by either direct cell-cell contact or the release of membrane fragments or microparticles from dead or dieing cells. Initially, it was observed that B-cells were responsible for the transfer of MHC II molecules to PMNs during PBMC co-culture experiments. Upon further investigation, however, a variety of syngeneic and xenogeneic cell types were effective donors of membrane lipids and proteins (Figures 5.3-5.6). There was no significant difference in the efficiency with which PMNs acquired MHC Class II protein from necrotic and apoptotic cells (Figures 5.3 and 5.4) but there did appear to be a relationship between the level of protein expression on a donor cell and the efficiency with which the protein was transferred to PMNs. For example, all T-cells express CD3 but only a subpopulation express MHC II protein at a relatively lower level. A much greater transfer of CD3 (Figure 5.5) than MHC II (Figure 5.4B) from bovine T cells to PMN was observed. The apparent lack of specificity in the passive transfer of membrane proteins implicates a mechanism that does not rely upon specific recognition signals for either protein uptake or insertion into the PMN plasma membrane. The binding of microparticles to the PMN surface and the subsequent fusion of these microparticles with the plasma membrane could explain this non-specific process.

Conditions that enhance the generation of microparticles might then be expected to enhance the passive transfer of integral membrane proteins. Cellular microparticles are shed from the plasma membrane of virtually all cell types when cells are subjected to a number of stress conditions, including apoptosis [27]. It was consistently observed that that the passive transfer of membrane proteins was most

efficient when donor cells were either apoptotic or necrotic. Cell survival assays indicated that bovine B-cells survived for a very short time in culture, providing a likely explanation for their role in MHC II transfer during PMN co-culture with PBMCs (Figure 5.4). In contrast, MHC II transfer was limited during PMN co-culture with ovine Clone 2 B-cells (Figure 5.4B) but TUNEL analysis revealed a low level of spontaneous apoptosis in this B cell line (data not shown). Finally, a more efficient CD3 transfer from bovine T cells to PMNs after T cell apoptosis was induced by gamma-irradiation was observed (Figure 5.5). It was also possible to transfer integral membrane proteins to PMNs by using the plasma membranes from physically disrupted cells (Figure 5.4B). Thus, it may be that microparticles, as well as a variety of other cell membrane fragments, can attach to the surface of PMNs. Apoptotic and necrotic cells, however, transferred MHC II molecules more efficiently than isolated cell membranes. Thus, integration of passively acquired membrane proteins into the PMN plasma membrane may be limited by the type of transfer vehicle which carries the integral membrane proteins (Figure 5.7).

The transfer of membrane fragments to immune cells may serve to either expand and/or regulate an immune response [217]. If PMNs gain antigen presentation capabilities when they acquire membrane proteins from dying APCs, then they might expand the immune response by presenting antigen to T-cells recruited to sites of inflammation. If, however, PMNs lack the appropriate co-signals to effectively activate T-cells then the results of PMN antigen presentation may be the elimination of self-reactive T-cells**,** the induction of tolerance, or the down-regulation of an active immune response. Alternatively, there is evidence that PMN can regurgitate processed antigen for other APCs to present during the induction of a specific

immune response [74]. Thus, antigenic peptides on passively acquired MHC II molecules may be transferred from PMNs to other antigen presenting cells. It is now critical to determine if the passive acquisition of a diverse array of integral membrane proteins can alter the functional activity of bovine PMNs. We are presently characterizing the mechanism by which PMNs passively acquire membrane proteins and investigating the functional consequences of this protein transfer.

CHAPTER 6

THE MECHANISM AND FUNCTIONAL CONSEQUENCES OF INTEGRAL MEMBRANE PROTEIN ACQUISITION BY BOVINE POLYMORPHONUCLEAR CELLS

6.1 Abstract

We have previously shown that bovine PMNs have an impressive capacity to passively acquire membrane fragments and associated integral membrane proteins from apoptotic and necrotic cells. The present study investigated the mechanism by which this integral membrane protein transfer occurs. We observed with confocal microscopy that microparticles (MPs) shed from fluorescently labelled blood leukocytes attached to the surface of PMNs. With time, MPs incorporated into the PMN plasma membrane and fluorescently labelled proteins diffused throughout the membrane. We also observed the transfer of cytoplasmic proteins, contained within MPs, from donor cells to PMNs. The functional consequences of PMN passive membrane protein acquisition were also investigated. Passive transfer of membranes from an adenovirus permissive cell line to PMNs significantly increased adenovirus infection of PMNs. Furthermore, passive transfer of ovine MHC II molecules enabled PMNs to induce xenoreactive T-cell proliferation and cytokine expression. In conclusion, the passive transfer of functional membrane proteins might have major implications for the immunological role of PMNs recruited to sites of inflammation.

6.2 Introduction

Research continues to reveal that leukocytes may perform diverse immune functions. This functional diversity at the cellular level creates extensive redundancy and a complex communication network within the immune system. The most important role of PMNs in innate immunity may be the clearance of immune complexes, phagocytosis of opsonized particles, and the release of inflammatory mediators. Recent research has revealed, however, the possibility that PMNs are functionally much more diverse than previously imagined. For example, when stimulated in an appropriate manner, PMNs may be able to acquire the functional characteristics of an antigen presenting cell (APCs) [208]. The present investigation examines the functional potential of bovine PMNs following the passive acquisition of integral membrane proteins from necrotic or apoptotic cells.

It was previously demonstrated that bovine PMNs rapidly acquire membrane fragments and immunologically relevant integral membrane proteins, such as MHC class II and CD3, from cells undergoing apoptosis or necrosis (Whale et.al. 2005, submitted). The phenomenon of membrane/protein transfer between cells is not a new finding, with several examples of such transfer between immune cells described in the literature [134,138-141,143,144]. T-cells, for example, can acquire immune complexes and other membrane proteins from APCs in a process dependent upon T-cell activation [138]. This exchange of membrane proteins was not limited to T-cells but also included the transfer of membrane proteins to B-cells, dendritic cells, macrophages and basophils. Furthermore, passively acquired membrane proteins may alter the function of recipient cells since T-cells that acquire MHC II/peptide complexes can either inhibit [138-141] or activate *in vitro* antigen-specific T-cells responses[142]. Thus, the functional consequences of membrane protein

transfer require further investigations, especially since this phenomenon may have implications beyond the regulation of immune responses. For example, the passive acquisition of an EBV viral receptor protein (CD21) by NK cells can confer susceptibility to EBV infection [145]. Thus, integral membrane proteins can retain their function following transfer from one cell to another.

Several mechanisms have been proposed by which protein may be transferred between immune cells. Transfer can involve an intimate interaction between cells at an 'immunological synapse' where tight junctions are formed between adhesion molecules. Recent electron microscopy of a CD8 T-cell/target cell immunological synapse revealed that a physical bridge formed between the cells to facilitate the lateral diffusion of proteins and lipids between fused membranes [146]**.** Dendritic cells use a different mechanism to physically sample extracellular or intracellular membrane/protein fragments from live cells [134] but other cells have also been shown to acquire membrane proteins from dead or dying cells [134]. Our investigations revealed that bovine PMNs acquired membrane proteins from apoptotic or necrotic cells and this transfer appeared to require close cell contact. We hypothesized that microparticles (MPs) shed from dying cells might mediate membrane protein transfer to PMNs. MPs, also referred to as microvesicles, are fragments shed from the plasma membrane blebs of virtually all cell types when cells are submitted to a variety of stress conditions, including apoptosis, and when cells are stimulated or activated [131]. There is increasing evidence that MPs can interact with adjacent and distant cells and may function as vectors for the intercellular exchange of biologic information.

Mammalian cell membranes are characterized by an asymmetric distribution of aminophospholipids with phosphatidylserine (PS) and phosphatidylethanolamine sequestered in the inner (cytoplasmic) leaflet and sphingomyelin and phosphatidylcholine sequestered in the outer (exoplasmic) leaflet [149]. When cells are exposed to apoptogenic stimulation, there is a collapse of this membrane asymmetry [121] and MP release is an integral part of the membrane-remodelling process. Since PS has procoagulant qualities, its translocation to the exoplasmic side of the cell membrane was initially thought to play a role in hemostatic control [150]. However, it has been proposed that PS may also function as a recognition determinant to target activated or apoptotic cells to phagocytes [151]. This hypothesis is based on the observation that the level of MP release into culture supernatants correlates with the degree of cellular apoptosis [152]. MPs, which are heterogeneous in size (0.05 - 1μm), protein and lipid composition, may also serve to transfer membrane proteins amongst cells. For example, MPs can carry adhesion molecule complexes [153] and passive acquisition of these complexes can alter cell signalling and adhesion [148]. Furthermore, MP transfer of the chemokine receptor CCR5, which acts as a co-receptor for human immunodeficiency virus (HIV), to non-CCR5 expressing cells confers susceptibility to HIV infection [154]. MPs may also transfer genetic information since tumour cell-derived MPs have been used to transfer oncogenes and induce tumour formation [158]. The diversity of MP functions and the observation that they were released from apoptotic cells led us to hypothesize that MPs may serve as the vehicle for the transfer of integral membrane proteins between apoptotic cells and bovine PMNs.

The aim of the present investigation was to elucidate the mechanism of passive membrane and protein transfer to bovine PMNs, as well as to determine the functional consequences of such protein acquisition. Using confocal microscopy, we were able to visualize MP formation by fluorescently labelled bovine PBMC and monitor MP attachment and subsequent fusion with PMNs. Viral infection and antigen presentation studies were used to determine if passively acquired proteins were functional and could confer new biological properties to recipient PMNs.

6.3 Material and Methods

6.3.1 Culture Media and Reagents

All cell cultures were carried out in AIM-V Serum Free Lymphocyte Medium (Gibco-BRL, ON, Canada) containing 20% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco-BRL) and 50 μM 2-mercaptoethanol (2-ME; Biorad, ON, Canada). This media is referred to as "complete AIM-V medium" unless otherwise stated.

6.3.2 Purification of Blood Leukocytes

Blood was collected from the jugular vein of male or female Holstein cattle (age > 12 months) using 0.3% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, ON, Canada) as an anticoagulant and PBMCs and PMNs were purified as described previously (Chapter 5). Cells isolated by these methods were consistently >98% viable as determined by trypan blue dye exclusion and contained less than 4% mononuclear cells. No attempt was made to differentiate between PMN and eosinophilic granulocytes, but the latter were usually present in low numbers (range = 2-10%).

6.3.3 Cell Lines

An ovine B-cell line (Clone 2; Cl2) was cultured as previously described [212] and green fluorescent protein expressing B cells (Cl2-GFP) were generated by using a retroviral vector to transduce the GFP gene [218]. The 293 cell line (ATCC# CRL-10852) was cultured in Dulbeco's minimal essential medium supplemented with 10% FBS (Gibco-BRL) and 293 cells were split 1:4 and passaged every 3-4 days.

6.3.4 Xenoreactive T-cell Populations

Bovine PBMC (10×10^6) were co-cultured 10:1 with γ-irradiated (11000 rads) ovine Cl2 cells in each well of a 6-well plate (Corning Inc., NY, USA). After 5 days, PBMC cultures were split 1:3 into fresh media supplemented with 2ng/ml recombinant human IL-2 (R&D Systems Inc., MN, USA). Cultures were again split 1:3 after 3 days and transferred to fresh media with IL-2 added for another 3 day culture. This xenostimulation procedure, with γ-irradiated Cl2 cell stimulation and IL-2 dependent T-cell expansion, was repeated once more before performing a mixed lymphocyte proliferative response (MLR) assay. Following the second xenostimulation, cells in the culture were >99% bovine $CD3^+$ T-cells and these cells developed a strong lymphoproliferative reaction (LPR) following addition of γ-irradiated Cl2 cells.

6.3.5 Biotin, PKH and Oxidative Burst Fluorescent Labelling

To track transfer of cellular membranes, cells were labelled with the lipophilic PKH 26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) according to the manufacturer's instructions. Similarly, to monitor the transfer of proteins, viable cells were surface labelled with EZ-Link Sulfo-NHS-Biotin (Pierce, IL, USA) according to the

manufacturer's instructions. Biotinylation of surface proteins was confirmed using flow cytometry to analyze streptavidin-fluorescein isothiocyanate (SA-FITC) binding. The compound 2`,7`-dichlorofluorescein-diacetate (DCFH-DA; Sigma-Aldrich) was used to fluorescently label PMNs since DCFH-DA specifically reacts with hydrogen peroxide. PMNs (1ml @ $1x10^7$cells/ml) were incubated with 0.1 mM DCFH-DA for 20 minutes and then washed twice with $Ca^{++}Mg^{+}$-free PBS. This procedure labelled over 99% of PMNs with green fluorescence. PKH is a lipophilic fluorescent dye that stably inserts into the plasma membrane and can be used to visualize the formation and transfer of MPs between cells. In contrast, biotin can be used to covalently label the extracellular domains of a broad variety of membrane proteins and following this labelling it is possible to monitor the transfer of membrane proteins made visible by labelling with SA-FITC. The non-fluorescent, non-polar dye DCFH-DA is able to cross PMN membranes and within the cell it is transformed by esterases into a polar molecule which cannot diffuse out of the cell. In the presence of H_2O_2, this polar DCFH is then converted to DCF, which fluoresces green and provides a PMN-specific marker. Bovine PBMCs were labelled with PKH-26 or biotin-SA-FITC prior to co-culture with PMNs which were either unlabeled or stained with PKH or DCFH-DA.

6.3.6 Transfer of Cell Membranes to Bovine PMNs

To achieve a rapid and consistent membrane protein transfer from 293-cells and Cl2 B-cells to bovine PMNs, the donor cells were lysed before co-culture with PMNs. This method of membrane protein transfer also eliminated potential contamination of PMNs with other cell populations. Donor cells (1×10^7 cells/ml) were sonicated for 2 x 7 seconds using a microtip vibrating at 20 kHz and amplitude of 40% (VibraCell ™

Sonicator, Betatex Inc.). Lysed cells were vigorously pipetted to disperse any clumps, before centrifuging for 3 minutes at 216 x g to remove intact cells. Membrane co-cultures with bovine PMNs were carried out for 24h with the cell ratio of 1 PMN: 3 'lysed cell equivalents'.

6.3.7 Construction of Recombinant Bovine Adenovirus BAV304 and Infection of Cells

A recombinant bovine adenovirus type-3 (BAdV-3) vector was constructed which contained the GFP gene in the E3 region (BAV304) [219]. BAV304 was added to 293-cell cultures, PMNs alone, PMNs co-cultured with lysed 293-cell membranes, or bovine PBMCs. Cultures were incubated for 48h before GFP expression was quantified with a flow cytometer. The detection of GFP expression in PMNs following BAV304 infection was restricted to viable PMNs by using propidium iodide (2.5 μg/ml) staining to exclude dead cells.

6.3.8 Flow Cytometry

The monoclonal antibody (mAb) specific for a conserved epitope on the alpha chain of the bovine ortholog of HLA-DR (Clone TH14B) was from VMRD (Pullman, WA, USA). Fluorescein isothiocyanate (FITC)-conjugated, isotype-specific goat anti-mouse immunoglobulin (Ig) antibodies were purchased from Southern Biotechnology (Birmingham, AL, USA). The level of specific mAb binding was quantified by subtracting the percentage of cells that reacting with an isotype-matched, irrelevant mAb (Cedar Lane Laboratories Ltd., Ontario, Canada). The irrelevant mAb was used at the same concentration (5μg/ml) as the relevant mAb. Cells were fixed in 2%

paraformaldehyde and stored in the dark at 4°C until data acquisition and was performed with a FACS Calibre flow cytometer (Becton Dickinson, CA, USA) and data analysis was performed using the Cell Quest program. This cell labelling and analysis protocol was used to confirm that significant amounts of lysed Cl2 cell membranes were acquired by bovine PMNs following co-culture.

6.3.9 MLR Assays

An MLR assay was used to assess responses of a xenoreactive T-cell population to Cl2 B-cells and to determine if PMNs could function as APC following passive acquisition of MHC II proteins from Cl2 membranes. Xenoreactive T-cells (5×10^4 cells/well) were co-cultured with either γ-irradiated Cl2 B-cells (1000 cells/well), γ-irradiated PBMCs (11000 rads, 5×10^4 cells/well), or PMNs incubated with or without Cl2 membrane fragments (5×10^4 cells/well). Cells were added to triplicate cultures of T cells in 96-well plates (Corning Inc.) and cultures were incubated for 90 hours at 37°C in a humidified atmosphere with 5% CO_2 before the addition of 0.4 μCi/well [methyl-3H] thymidine (Amersham Pharmacia Biotech) during the final 6 hours of culture. Cultures were harvested onto unifilter glassfiber microplates (Perkin Elmer, Woodbridge Ontario, Canada) and incorporation of 3H-thymidine was measured using a microplate scintillation and luminescence counter (Top Count, Canberra Packard, ON, Canada). Data are expressed as a stimulation index (SI = cpm with Cl2 B-cells or other stimuli/cpm for T-cells cultured in medium alone) and all assays were performed with xenoreactive T-cell populations generated from 6 animals.

6.3.10 Analysis of T-Cell Cytokine Gene Expression

Xenoreactive T-cells (5 x 10^6/well), specific for ovine Clone 2 B-cells, were plated in a 12 well plate (Corning/ VWR CanLab) and incubated for 2h, 4h, 8h or 12h with 5 x 10^5 γ-irradiated Cl2 cells, 1 x 10^6 syngeneic PMNs, or 1 x 10^6 syngeneic PMNs that had been incubated with membranes from 3 x 10^6 lysed Cl2 cells. T cell cultures were collected at each time point, washed twice with PBSA, and then lysed with 1 ml Trizol (Gibco-BRL). Total RNA was extracted as described previously [220]. RNA concentration and quality were determined spectrophotometrically by measuring absorbance at 260 nm and purity was assessed using an Agilent 2100 Bioanalyzer with RNA 6000 Nano kits (Agilent Technologies Canada Inc., ON, Canada). The quality of total RNA was assessed by comparing the ratio of the area under the ribosomal peaks for 28S and 18S. Quantitative Real-Time PCR (qRT-PCR) was conducted using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen) on the Bio-Rad iCycler (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) as indicated in manufacturer's protocol. Primers for sense and anti-sense strands of selected genes (Table 6.1) were designed using Clone Manager 7 (Scientific & Educational Software, 600 Cary, NC., USA) and PCR products were sequenced to confirm the specificity of the primers (data not shown). The qRT-PCR analysis for each cytokine was performed in triplicate using RNA isolated from three T-cell populations and C_t-values were normalized with respect to actin C_t-values. A Melt Curve was performed to ensure that any product detected by the iCycler was specific to the desired amplicon. The data is reported as ΔC_t-values which represents the number of PCR cycles required to detect fluorescence for a gene of interest subtracted from the number of PCR cycles required to detect fluorescence from the actin transcript within the same sample.

Table 6.1: Primers to amplify bovine cytokines

Bovine Gene	Accession Number[a]	Primer Direction	Primer Sequence (5'-3')
IL-2	AF535144	Forward	TCCTGCATCCTCTACAAGTG
		Reverse	AGACATCCTGGGCTTGAAAC
IL-4	TC269418	Forward	ACGCTGAACATCCTCACAAC
		Reverse	CGCCTAAGCTCAATTCCAAC
IL-10	NM174088	Forward	GCTGTATCCACTTGCCAACC
		Reverse	CCAGGTAACCCTTAAAGTCATCC
TNFα	AF011926	Forward	GGCGGAGTGTAGGAAGTATC
		Reverse	CATCTGGAGGAAGCGGTAG
IFN-γ	M29867	Forward	CCTCCTTGGGACCTGATCATAACAC
		Reverse	CCAAAAGCCCACAGAGCAGTAAAG

[a]**(www.ncbi.nlm.nih.gov)**

6.3.11 Confocal Laser Scanning Microscopy (CLSM)

CLSM analyses were performed with an MRC 1024 confocal laser scanning microscope (Bio-Rad, Hemel Hempstead, UK) attached to a Microphot SA microscope equipped with an oil immersion 60x 1.4 numerical aperture plan apochromat lens (Nikon, Japan), and a 10x eyepiece lense. High magnification pictures were taken using the same lens with a 3x zoom. For each condition, 20 µl of cell co-cultures were placed on a glass slide with a glass coverslip before mounting the slide on the viewing stage.

6.4 Results

6.4.1 PBMC Microparticle Formation and Adherence to PMNs

To test the hypothesis that MPs were shed from PBMCs and then attached to and subsequently fused with PMN membranes, confocal microscopy was used to analyze interactions between these two cell populations. It was known from previous findings that PMNs could acquire membrane fragments and protein from isolated bovine PBMCs

very rapidly (within 40 minutes) and this acquisition could occur at 4^{o}C (Whale *et.al.* 2005, submitted). Using a combination of staining techniques to identify donor and recipient cells, the formation and release of MPs from bovine PBMCs (Figure 6.1A), and subsequent attachment of MPs to the surface of PMNs was observed (Figure 6.1B). Figure 6.1C shows a PKH labelled PMN (red) in close proximity to a biotinylated and SA-FITC labelled PBMC (green) and a much smaller MP (green). These observations support the conclusion that MPs are shed from bovine PBMCs and that MPs can mediate the transfer of both membranes and integral membrane proteins from PBMCs to PMNs. Direct cell-cell contact may also play a role in this process (Figure 6.1C).

6.4.2 Fusion of MPs with PMN Membranes

To understand the process of passive membrane protein acquisition it is critical to determine if MPs integrate into the PMN plasma membranes. If MPs simply attach to the PMN surface then it is unlikely that passively acquired proteins would confer new cellular functions beyond possible changes in cell adherence properties [153]. Co-culture of labelled PBMC with PMNs resulted in detectable transfer of MPs to the PMNs surface (Figure 6.2A) and lipophilic labelling with PKH revealed diffusion of MP membrane throughout the PMN plasma membrane within 20-30 minutes of co-culture (Figure 6.2B). To confirm that PMNs were the recipients of MP membrane proteins, this experiment was repeated with PMNs labelled with PKH (red) and PBMC surface proteins labelled with biotin and SA-FITC (green, Figure 6.2C). It again appeared that acquired membrane proteins were diffusing throughout the PMN plasma membrane (yellow). Finally, PMNs

were labelled internally with DCFH-DA (green) to facilitate better visualization of PKH-labelled PBMC membrane (red) diffusion throughout the PMN plasma membrane (Figure 6.2D). Collectively, these observations support the conclusion that passively acquired membranes and membrane proteins integrated into the PMN plasma membrane. Previous flow cytometric experiments had suggested PMN passive acquisition of membrane proteins from PBMC increased with time (Chapter 5). Confocal microscopy was used to examine this process in more detail and to determine if the integration of PBMC membranes and integral membrane proteins increased

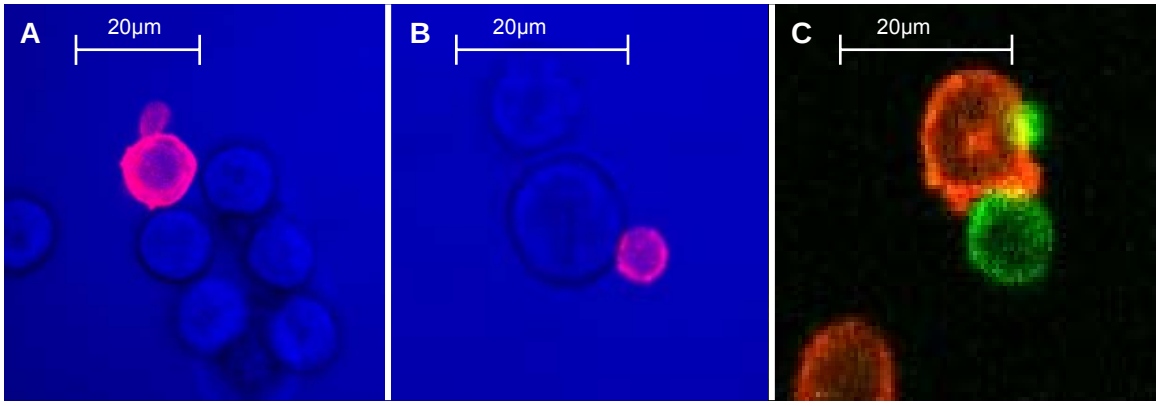


Figure 6.1. Formation and release of MPs from PBMC and MP attachment to bovine PMNs. (A) MP formation and detachment from PKH dye (red) labelled PBMCs was observed with confocal microscopy. (B) Attachment of a MP shed from a PBMC to the surface of an unlabelled PMN. (C) PBMCs labelled with biotin-SA-FITC (green) shed MPs (green) which then attached to the surface of a PKH dye (red) labelled PMN. Close cell-to-cell contact between PBMC (green) and PMN (red) was also observed.

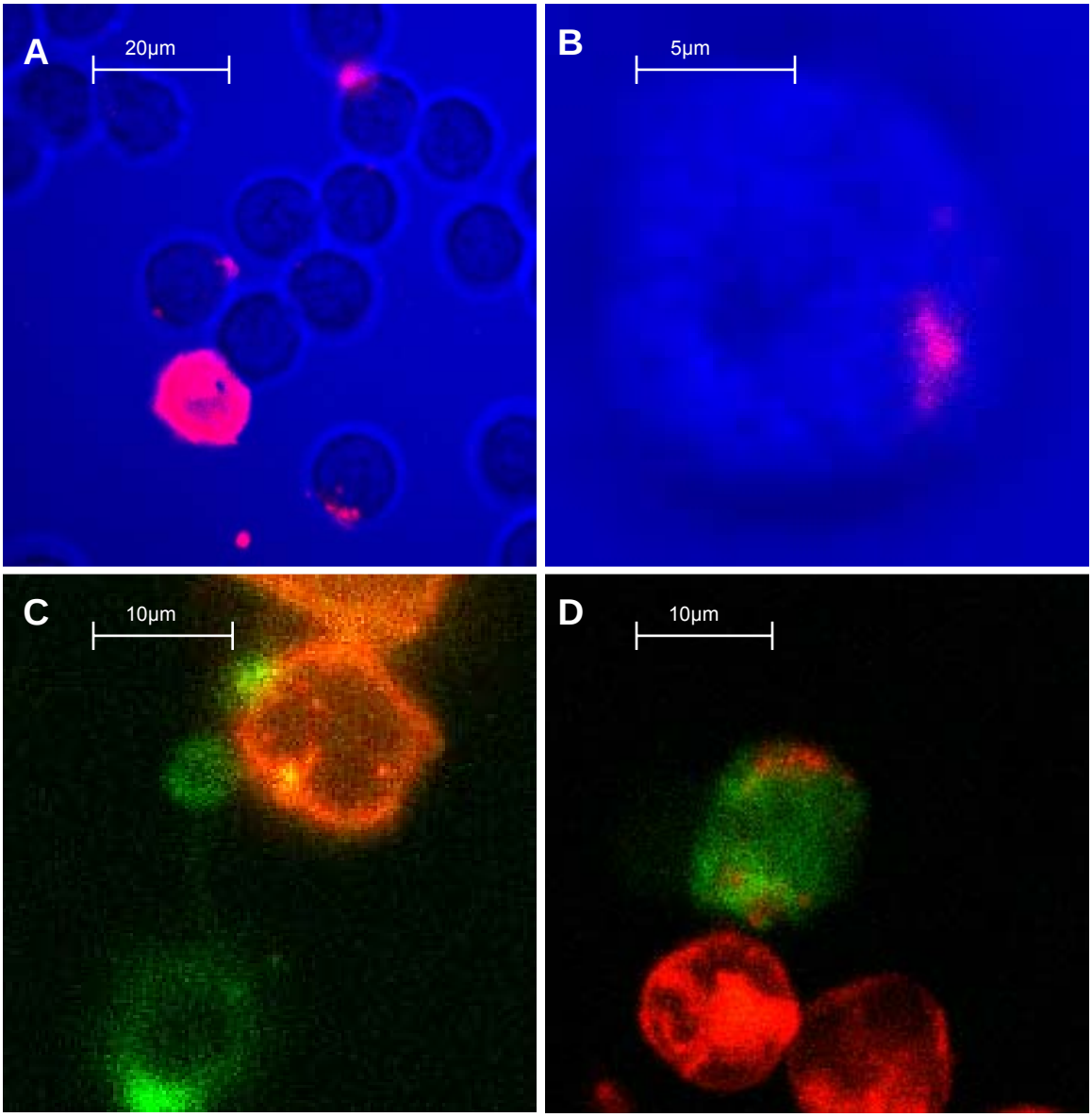


Figure 6.2. Integration of passively acquired membranes and membrane proteins into the PMN plasma membrane. (A) Attachment of both PBMC (red) and shed MPs (red) to the surface of PMNs (no label). (B) Diffusion of lipophilic PKH label (red) throughout plasma membrane of PMN (unlabelled).(C) Attachment of PBMC-derived MPs containing biotin-SA-FITC labelled membrane proteins (green) to the surface of PKH labelled PMNs (red). Yellow fluorescence may reveal integration or mixing of PBMC-derived membrane proteins (green) within the PMN plasma membrane (red). (D) PMN labelled with DCFH-DA (green cytoplasm) display diffuse surface labelling with PKH labelled PBMC membranes (red).

over time. Very little membrane attachment was detected following a brief co-culture (Figure 6.3A) but membrane attachment did increase with time (Figure 6.3B and C). Furthermore, following a 24 h co-culture of PMNs and PBMC there was extensive dispersion of labelled PBMC membranes throughout the PMN plasma membrane (Figure 6.3D). These observations support the conclusion that there is a time-dependent integration of passively acquired membranes and integral membrane proteins into the PMN plasma membrane.

6.4.3 Transfer of Cytoplasmic Protein to PMNs

The observation that MPs fused with PMNs raised the possibility that either cytoplasmic proteins or genetic material (DNA or RNA) contained within MPs may also be transferred to PMNs. An ovine B-cell line, expressing cytoplasmic GFP (Cl2-GFP), was used to determine if detectable levels of cytoplasmic proteins could be transferred by MPs to a recipient cell. Following γ-irradiation, Cl2-GFP cells formed (Figure 6.4A) and released (Figure 6.4B) MPs which contained detectable levels of GFP. It was difficult to identify recipient PMNs with a strong green fluorescent signal following attachment of GFP-labelled MPs but individual PMNs were found that contained both discreet and diffuse GFP staining (Figure 6.4C). Discreet foci of green fluorescence may indicate endocytosis of GFP containing MPs and diffuse green fluorescence might indicate either release of GFP from the endocytic vesicle or a direct release of GFP into the PMN cytoplasm following fusion of MPs with PMNs.

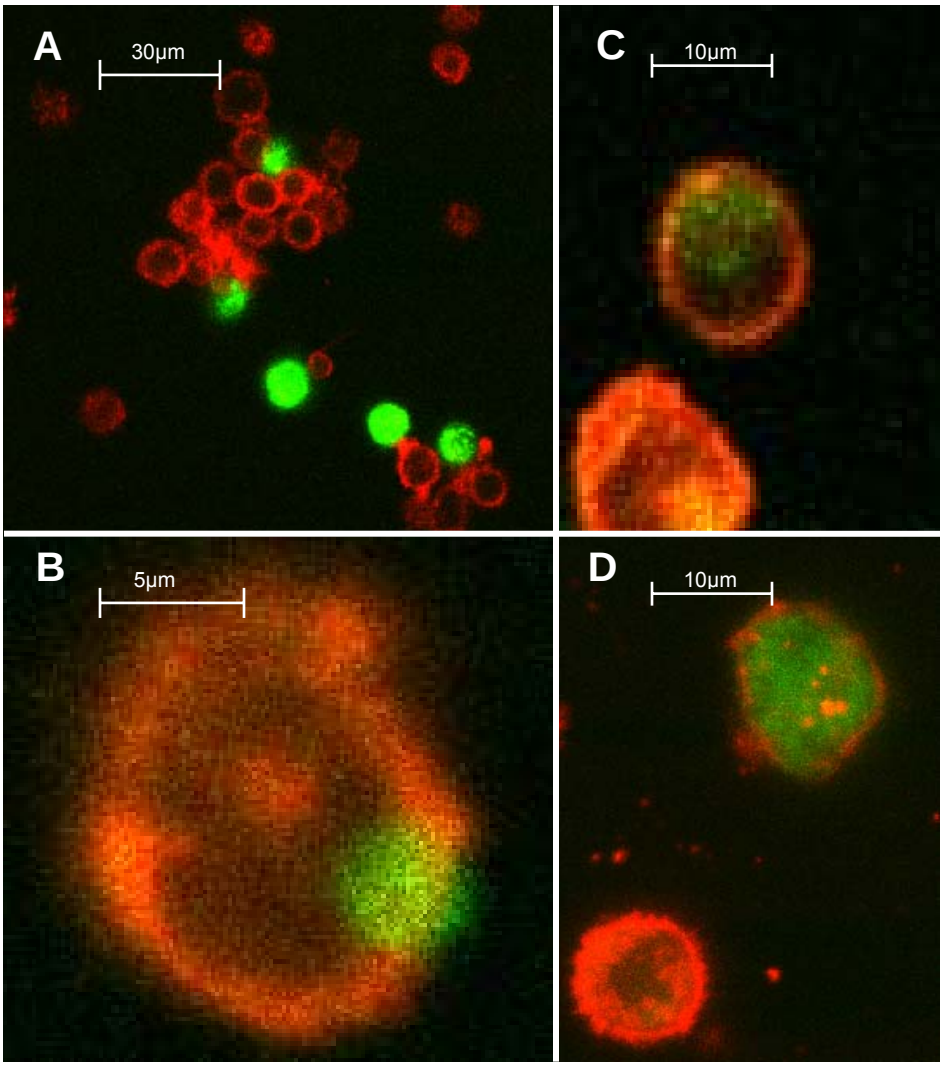


Figure 6.3. PMN acquisition of PBMC membranes and integral membrane proteins increases with time. (A) No detectable transfer of PBMC membranes (red) to PMNs (green, DCFH-DA label) was observed when co-culture was less than 5 minutes. Following co-culture for (B) 20-30 min or (C) 2h, PMNs (red, PKH label) had acquired increasing amounts of biotin-labelled protein from PBMCs (green). Following co-culture for 24 h (D) extensive integration of PKH-labelled PBMC membranes was observed on the surface of DCFH-DA-labelled PMNs (green).

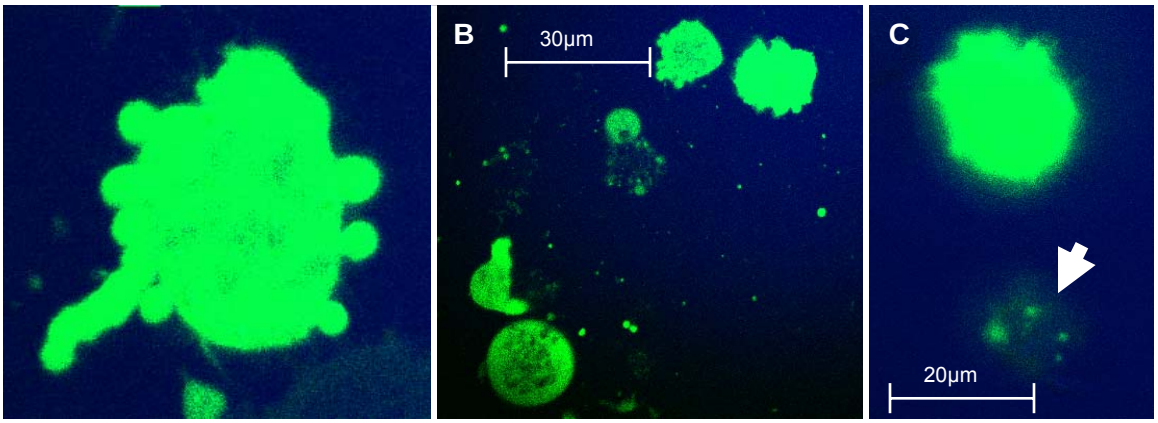


Figure 6.4. MP transfer of cytoplasmic proteins to PMNs. (A) Generation of MPs containing GFP following γ-irradiation of Cl2-GFP cells. (B) Release of MPs containing detectable GFP fluorescence from γ-irradiation of Cl2-GFP cells. (C) Binding of MPs containing GFP to a PMN (unlabelled) and diffusion of GFP within the cytoplasm of a PMN (arrow).

6.4.4 Passively Acquired Proteins Retain Their Function

Confocal microscopy provided evidence for integration of passively acquired proteins within the plasma membrane of PMNs. Therefore, we used two different assays to determine if passively acquired proteins altered PMN function. The first assay was based on PMN infection with recombinant BAdV-3 which expressed GFP (BAV304). This assay allowed a direct measurement of the number of infected PMNs. BAV304 infection of PMNs with an MOI of 1 results in detectable GFP transgene expression in approximately 7-15% of cells (Figure 6.5A). In contrast, 293-cells are more permissive to BAdV-3 infection and GFP transgene expression is detectable in all cells at 48h post-infection (MOI=1, Figure 6.5A). Although cellular receptors for BAdV attachment have not been identified, it is well accepted that viral entry is protein mediated [221]. Therefore, PMNs were incubated with and without cell membranes from lysed 293-cells, and then compared BAdV-3 infection through GFP-transgene expression. Co-incubation of PMNs with cell membranes from lysed 293-cells increased the number of cells with detectable GFP transgene expression by approximately 3-fold (Figure 6.5A). Furthermore, this increased frequency of GFP^+ PMNs was accompanied by an increased level of GFP expression within individual cells (Figure 6.5B). The specificity of 293 membrane protein transfer to PMNs was evaluated by co-culturing PMNs with bovine PBMCs using conditions known to transfer membrane proteins. Bovine PBMCs are not infected by BAV304 (data not shown) and PMN expression of GFP was not significantly altered when infected with BAV304 following co-culture with PBMCs (Figure 6.5A). These observations support the conclusion that passive transfer of a functional integral

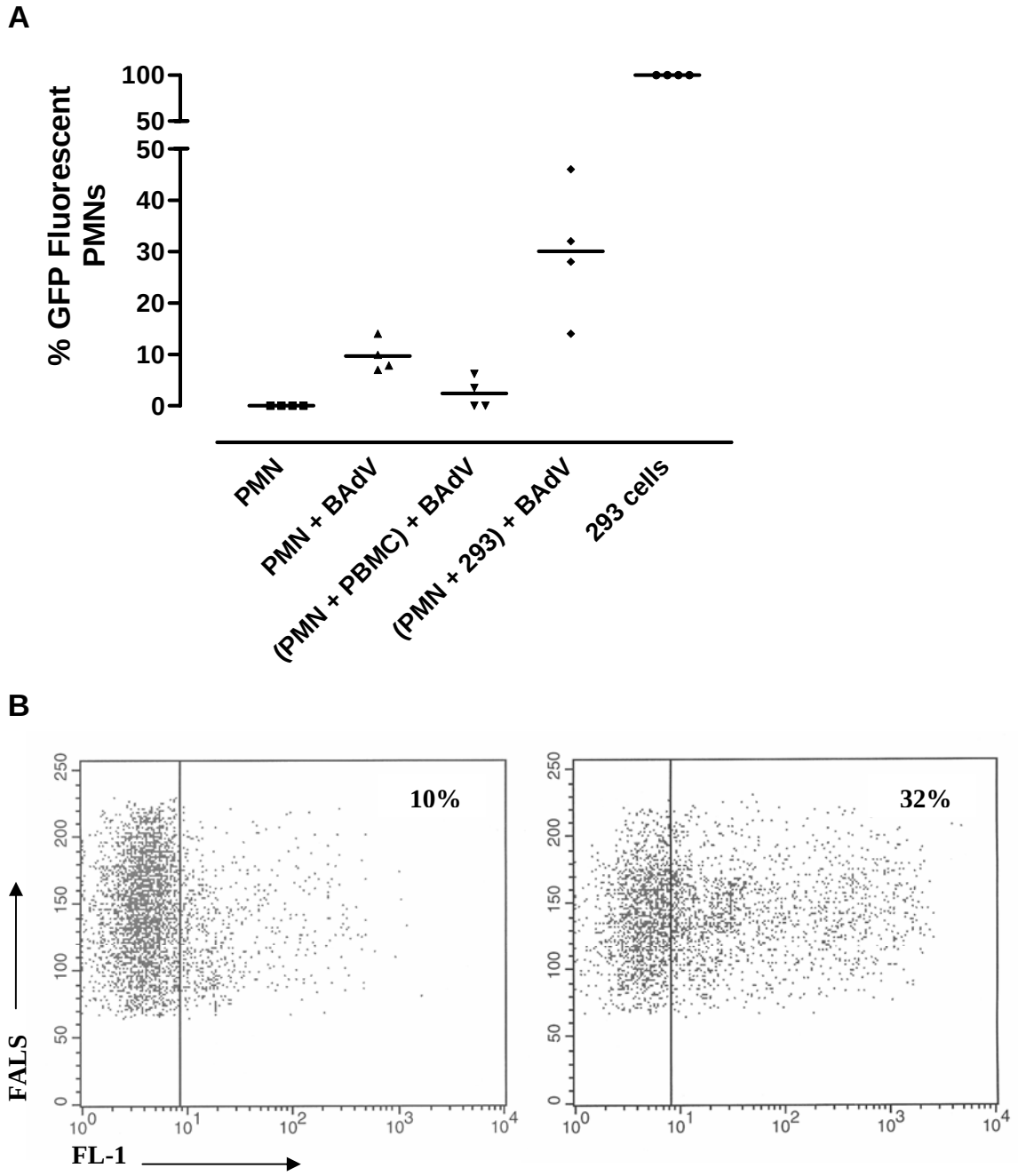


Figure 6.5: Infection of bovine PMNs with recombinant BAdV-3 expressing GFP protein (BAV304). (A) GFP expression in PMNs was measured at 48 hours post-infection using flow cytometry. Uninfected PMNs were used as the negative control (PMN) and the BAdV-3 permissive cell line (293 cells) was the positive control. PMNs were infected directly with BAV304 (PMN + BAdV) or infected following co-culture of PMNs with either 293 cell membranes ((PMN + 293) + BAdV) or non-permissive leukocytes (PMN + PBMC) + BAdV). Data presented are values for PMNs isolated from 4 individual animals (bar=median value for each group). The unpaired, non-parametric Mann-Whitney T-test revealed a significantly ($P = 0.03$) increased percentage of PMNs were infected following incubation with 293 cell membranes ((PMN + 293) + BAdV) when compared to BAV304 infected PMNs alone (PMN + BAdV). (B) Representative FACS dot-plot profiles showing GFP expression at 48h post-BAV304 infection of PMNs alone (left panel) or bovine PMNs pre-incubated with 293-cell membranes (right panel).

membrane protein from 293-cells to bovine PMNs significantly altered the capacity of PMN to be infected by BAdV-3.

A second, more specific method was then used to analyze the functional consequences of passive membrane protein acquisition by PMNs. Xenoreactive bovine T-cell populations were generated using the ovine Cl2 B-cell line and these T-cell populations were then used to determine if passively acquired ovine MHC II molecules could induce antigen-specific T-cell activation. The transfer of membranes from lysed Cl2 B-cells to either syngeneic or allogeneic PMNs induced an equivalent antigen-specific proliferative response by the xenoreactive T-cell line (Figure 6.6A). The current model for an MLR is that alloreactive T-cells recognize genetically disparate MHC molecules with bound peptide in the groove [66]. If this model is correct then the present experiments confirm that bovine PMNs which passively acquire MHC II proteins (Figure 6.6B) can function as APCs for T-cells that have been previously activated by antigen. The MLR induced by PMNs was significantly less than that observed with γ-irradiated Cl2 B-cells. This may simply be due to the much lower level of passively acquired MHC II protein on PMNs (Figure 6.6B) or there may be qualitative differences in antigen presentation by PMNs. The passive transfer of co-stimulatory molecules with MHC II was not determined but we analyzed T-cell cytokine expression to determine if PMN stimulation of T-cells was qualitatively different from the T-cell activation induced by Cl2 B-cells. Expression of TH1-type (IFN-γ; TNF) and TH2-type (IL-4; 1L-10) cytokines was analyzed and IL-2 expression was also analyzed since this cytokine is important for T-cell proliferative responses. Cytokine expression analysis revealed that PMNs, following passive acquisition of ovine MHC II, induced

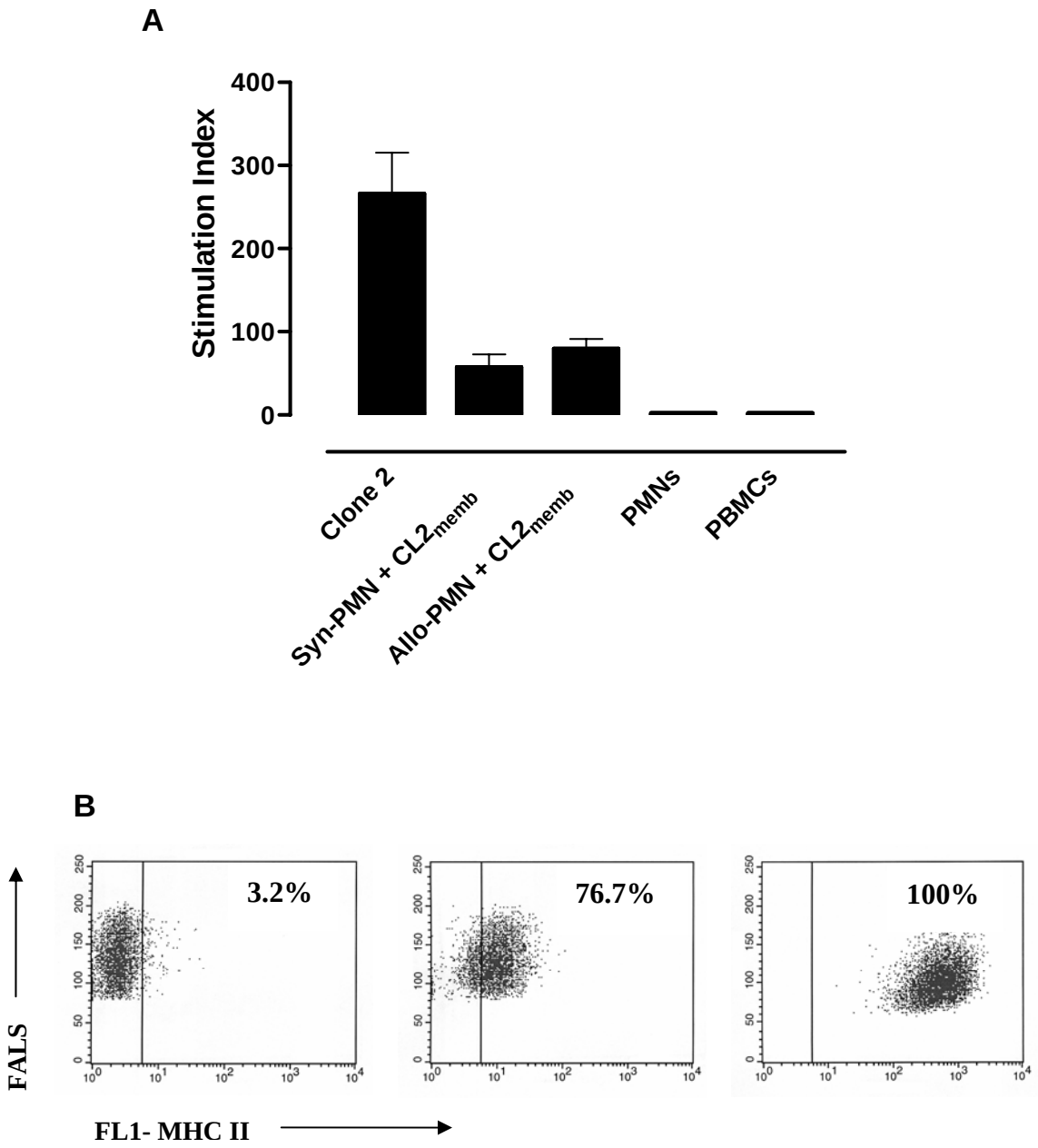


Figure 6.6: Bovine PMNs which passively acquire ovine MHC II induce proliferation of xenoreactive T-cells. (A) Proliferation of a xenoreactive T-cell line was measured following co-cultured with either γ-irradiated Clone 2 B-cells (Clone 2), syngeneic PMN incubated with Cl2 membranes (Syn-PMN + $CL2_{memb}$) or allogeneic PMN incubated with Cl2 membranes (Allo-PMN + $CL2_{memb}$), bovine PMNs alone (PMNs), or syngeneic PBMCs (PBMCs). Data presented are the mean and 1 SD of values from triplicate cultures of a T-cell line generated from one animal. Similar results were obtained with Cl2-specific T cell populations generated from 5 other animals. (B) A representative FACS profile of MHC II expression on bovine PMNs alone (left panel) or following PMN co-culture with membranes from lysed Cl2 cells (middle panel). The level of MHC II expression on CL2 B-cells is shown in the right panel.

T-cell expression of both TH1- and TH2-type cytokines (Figure 6.7). The onset, amplitude, and duration of cytokine gene expression varied significantly when PMN stimulation was compared to Cl2 B cells. Furthermore, as expected from MLR results (Figure 6.6A), PMNs which passively acquired ovine MHC II induced significantly less IL-2 expression than T-cells stimulated with Cl2 B-cells. Thus, PMN stimulation of activated, xenoreactive T-cells resulted in quantitative differences in cytokine gene expression but expression was significantly increased for all cytokines analyzed. When reading the results reported in Figure 6.7, remember that a lower ΔC_t-value indicates an increased level of expression of the gene of interest.

6.5 Discussion

There is increasing data to support the conclusion that the passive transfer of membranes and integral membrane proteins among immune cells is a general phenomenon and this exchange of biological material can have important functional consequences [134,138-141,143,144]. Few studies have explored the mechanisms of membrane/protein transfer in depth, although passive acquisition of protein from dying cells has been previously reported [134]. The present study clearly shows that MPs shed from fluorescently labelled PBMCs can attach and integrate into the PMN plasma membrane. It is important to note that all short-term PMN and PBMC co-cultures were incubated at 20-22^{o}C prior to viewing with confocal microscopy. This may be significant since membrane fluidity and fusion may be reduced at this temperature when

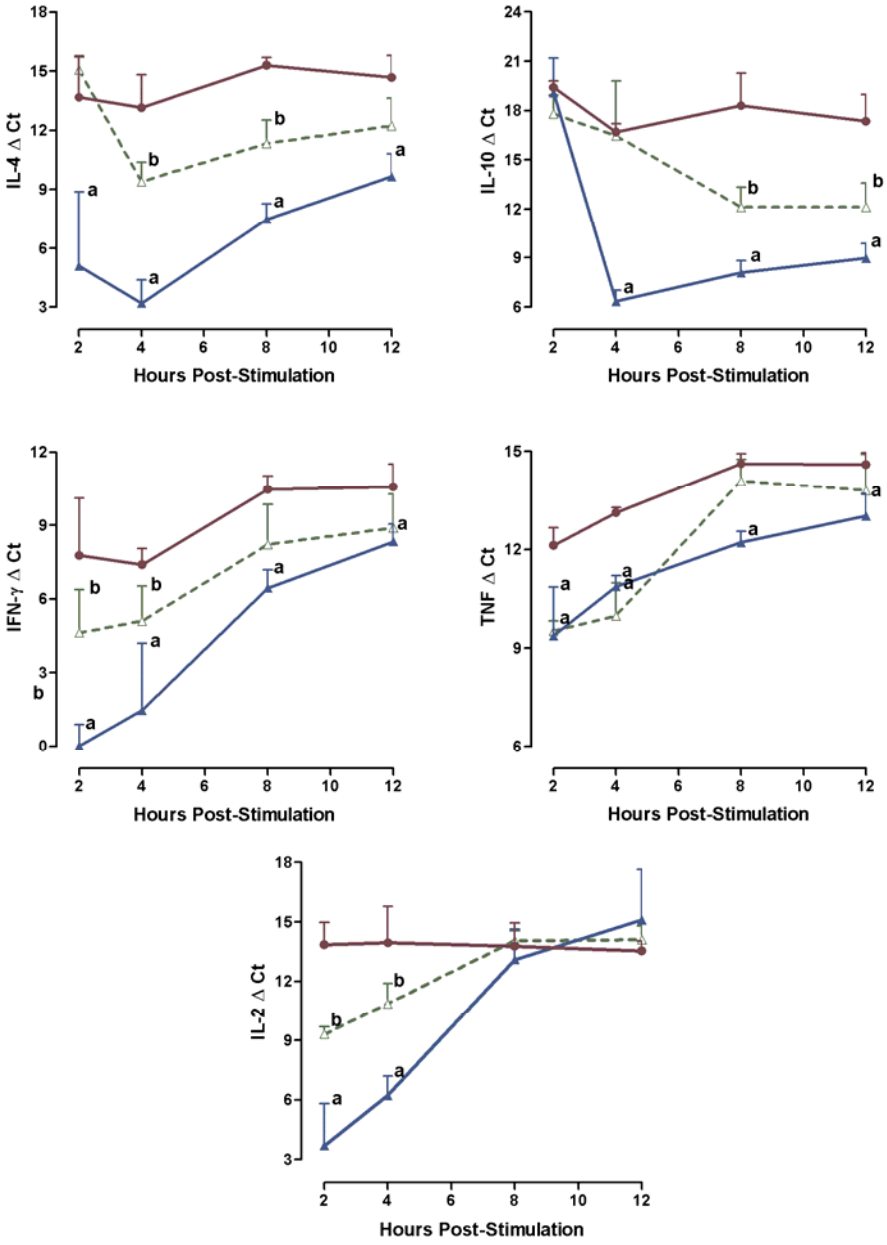


Figure 6.7. Bovine PMNs which passively acquire ovine MHC II induce cytokine expression in xenoreactive T-cells. Cytokine expression was measured using qRT-PCR and the level of cytokine expression is expressed relative to actin (ΔCt), therefore the lower ΔCt values are indicative of a higher level of gene expression. Xenoreactive T cells were stimulated with either γ-irradiated Cl2 cells (T + CL2 B-cells; solid triangle), syngeneic PMNs (T + PMN; solid circle), or syngeneic PMNs incubated with Cl2 B-cell membranes (T + PMN/CL2 Memb.; open triangle). T cell cultures were collected for RNA isolation following the indicated co-culture intervals and data presented are the mean and 1 SD for values from xenoreactive T cell populations derived from 3 animals. Superscripts indicate values that are significantly ($p < 0.05$) different from the T + PMN culture and data points with different superscripts differ significantly ($p < 0.05$) from each other.

compared to body temperature [215]. Thus, passive acquisition and fusion of membranes and integral membrane proteins with the PMN plasma membrane may be even more efficient at body temperature. It is also interesting to note that during the confocal studies there was no evidence of reciprocal membrane/protein transfer from PMNs to PBMCs even following a 24h co-culture at 37^{o}C (Figure 6.3D). Finally, the present investigation also provided evidence that MPs can transfer cytoplasmic protein between cells (Figure 6.4). In conjunction with previous data, which confirmed oncogene transfer between cells [158], we must now consider the possibility of RNA and cytoplasmic proteins transfer. Further studies will be required to determine if the magnitude of this cytoplasmic transfer is sufficient to alter the biology of the recipient cell.

Confocal microscopy provided substantial evidence that MP attachment to PMNs was followed by integration of passively acquired membranes and membrane proteins (Figures 6.2 and 6.3). A previous study demonstrated that MPs shed from leukocytes attached to the surface of epithelial tumour cells but the acquired proteins remained in segregated 'rafts' rather than diffusing throughout the recipient plasma membrane [148]. In contrast, both labelled lipids and biotin-labelled proteins from donor leukocytes appeared to diffuse throughout the plasma membrane of bovine PMNs. These observations were consistent with a full integration of membranes/proteins within the plasma membrane of the bovine PMN with no apparent restriction in their subsequent distribution. Furthermore, time course studies indicated that this integration can occur relatively rapidly. The attachment of MPs to the surface of bovine PMNs may explain a previous report that goat PMNs can passively acquire MHC II but this MHC II expression is dependent upon the methodology used to isolate the cells [80]. Centrifugation of goat PMNs through a density gradient was reported to remove surface MHC II molecules. A similar

phenomenon with bovine PMNs following centrifugation was observed here (Chapter 5). It may be that certain isolation procedures can remove leukocyte-derived MPs which contain MHC II molecules and are loosely attached to the PMNs surface. Thus, reports that surface molecules can be removed during a cell isolation procedure may need to be re-examined for potential passive acquisition of surface proteins from cells which are shedding MPs.

The passive acquisition of integral membrane proteins by bovine PMNs was of sufficient amplitude to significantly alter recipient cell biology (Figures 6.5 and 6.6). This research has shown that the passive acquisition and integration of functional membrane proteins altered viral infection of PMNs and enabled PMNs to activate antigen-specific T-cells. Increased viral infection of bovine PMNs was observed following their exposure to lysed cell membranes from 293-cells which are highly permissive to BAV304 infection (Figure 6.5). PMNs are not permissive to BAdV-3 replication (data not shown) and thus increased GFP-transgene expression cannot be interpreted as increased viral replication. This assay primarily provided a method to monitor viral uptake by PMNs and, therefore, provides indirect evidence for the passive transfer of a viral receptor protein from 293-cells to PMNs. These observations are consistent with previous reports that viral receptor proteins can be passively acquired and subsequently alter viral tropisms [145,154].

This study provided direct evidence that PMNs could passively acquire MHC II molecules which conferred antigen presentation capabilities upon the PMNs. In these experiments, the passive acquisition of ovine MHC II (Figure 6.6B) resulted in bovine PMNs inducing a significant MLR with antigen-specific T-cell populations (Figure 6.6A). This proliferative response was by primed T-cells, but specific T-cell activation

should still be dependent upon a functional MHC class II protein with antigen bound in its groove [66]. The requirement for co-stimulation signals may be much less for activation of primed T-cells and in this assay, co-stimulation would be dependent upon a functional cross-reactivity between ovine and bovine co-stimulatory molecules. Passive acquisition of ovine MHC II by bovine PMNs induced significantly lower levels of T-cell proliferation than Cl2 B-cells and this difference in T-cell responses was observed despite using greater numbers of stimulator PMNs than γ-irradiated Cl2 B-cells. Decreased T-cell activation may reflect the 40-fold lower density of passively acquired MHC II molecules on bovine PMNs (MFI = 13.8) than on donor Cl2 B-cells (MFI = 617) (Figure 6.6B). Alternatively, decreased PMN-induced T-cell responses may reflect either a lack of co-stimulatory molecule transfer or reduced cross-species activity of passively transferred ovine co-stimulatory molecules. The present assay was designed to clearly discriminate between potential endogenous MHC II expression and passively acquired MHC II molecules. This assay may not, however, be optimal for exploring the full potential of bovine PMNs to function as APCs following passive acquisition of MHC II molecules.

It has been postulated that the transfer of membrane fragments to immune cells may serve to either expand and/or regulate an immune response [217]. For example, macrophage antigen presentation is reduced following phagocytosis of apoptotic cell bodies but antigen presentation is enhanced following phagocytosis of necrotic cell bodies [83]. Thus, we examined in more detail the antigen presentation capacity of PMNs following passive acquisition of ovine MHC II. Cytokine gene expression analysis did not reveal a marked bias or deficiency in T-cell activation when PMNs were functioning as APCs (Figure 6.7). The magnitude and duration of gene expression was markedly

reduced when the T-cells were stimulated using PMNs + Clone 2 cell membranes as the APCs rather than with Clone 2 cells. However, the same pattern of gene expression was observed regardless of the stimulator population used. That is, Th1 cytokines are expressed earlier than Th2 cytokines, and following the induction of IL-10, Th1 cytokine expression was down-regulated. The relative levels of gene expression were compared to the relevant control of T-cells which had been co-cultured with purified PMNs. This control showed no induction of T-cell gene expression over time, and therefore revealed very low level cytokine gene expression in unstimulated T cells. Although PMNs were not potent APCs, one implication of the current *in vitro* studies may be that PMNs, at a site of inflammation, could further stimulate activated T-cells. The relatively large number of PMNs that can infiltrate sites of inflammation may compensate for their relatively poor antigen presentation by providing T-cells with an increased opportunity to interact with antigen. The efficiency and consequences of T-cell activation by PMNs may also depend upon which co-stimulatory molecules are passively acquired through the uptake of MPs. Further studies will be required to determine if the co-transfer of MHC II and co-stimulatory molecules has a significant impact on PMN antigen presentation.

In summary, these studies confirmed that bovine PMNs could rapidly acquire membrane fragments and their associated proteins through MPs which have been shed from dying cells. *In vitro* studies have also confirmed that these passively acquired membrane proteins retain their function and confer novel biological properties to recipient PMNs. The observation that passively acquired MHC II can be presented to activated T-cells provides a novel mechanism by which PMNs may play a role in both innate and adaptive immune responses.

CHAPTER 7

General Discussion and Conclusions

The mammalian immune system is complex, dynamic and diverse. Immunologists have wrestled with this complexity in an attempt to understand how defence against a massive number of highly adaptable pathogens is mounted. It could be argued that vertebrates can only exist thanks to an immune defence system whose repertoire matches that of invading viruses and microorganisms. Immunologists have conceptually divided the mammalian immune system into innate and adaptive immune responses. It seems, however, that the more that is discovered about how the immune system works, the less clear these functional distinctions are, and the more integrated immune responses become. In addition, immunological studies are revealing many redundancies in immune function, such as antigen presentation which was once thought to be the domain of a few specialized cell types.

PMNs are a very important component of the innate branch of the mammalian immune system as evidenced by the fact that no mammal can survive for more than a few days without them [13]. PMNs are often the first cell recruited to a site of inflammation to provide a rapid response to microbial invasion but evidence is emerging which suggests that PMNs may also play an important role in modulating adaptive immunity. Not only have PMNs been shown to express several important regulatory cytokines as outlined in chapter 1, the research described in this thesis demonstrates that bovine PMNs have an impressive capacity to rapidly acquire functional proteins from dead or dying cells.

The passive or active transfer and acquisition of membrane fragments and their associated integral membrane proteins amongst leukocytes (T-cells, B-cells, dendritic cells, monocytes and NK cells) has been an emerging topic of interest in recent years (outlined in Chapter 1). Furthermore, this transfer of membrane fragments and membrane proteins has been associated with a variety of interesting immunological phenomena, such as cross-presentation, T-cell antigen presentation and the transfer of viral tropism to cells which acquire viral receptor proteins. The current evidence strongly argues that PMNs should be included among the list of immune cells which can passively acquire functional proteins. The unique biology of PMNs has significant implications for the potential immunological implications of passive membrane acquisition and may also provide unique opportunities to exploit this phenomenon in areas such as the delivery of vaccine antigens.

Evidence for Passive Acquisition of MHC II by Bovine PMNs

Prior investigation that bovine PMNs could function as antigen presenting cells led to the observation that PMNs were preferentially targeted, when comparing all blood leukocytes, for infection by BAdV-3 raised a major issue concerning the efficacy of using BAdV-3 vaccine vectors. If PMNs could destroy a significant portion of the viral vaccine, the efficiency of vaccination could be substantially reduced, and would have to be addressed. However, previous studies had shown that BAdV-3 vaccine vectors could indeed stimulate strong immunity to the antigens they expressed [211]. It was therefore hypothesized that PMNs may participate in antigen presentation either directly to T-cells, or by an indirect pathway.

Initially the expression of MHC Class II, a key protein involved in antigen presentation, on bovine PMNs was investigated. These experiments confirmed that purified bovine PMNs were MHC II^- when isolated from whole blood but if purified PMNs were co-cultured with PBMCs then MHC II^+ expression was detected. Experiments were then conducted to determine whether the PMN isolation technique altered the level of MHC II expression on the surface of PMNs. As had been previously reported using caprine PMNs isolated with density gradient centrifugation [80], it was observed that following bovine PMNs isolation with centrifugation speeds of 1400 x g there was no detectable MHC II expression. There are no reports that centrifugation of cells at these speeds can shear off integral membrane proteins. Thus, the interpretation of these observations was that MHC II molecules were present on the freshly isolated bovine PMNs, but as loosely adhered MPs. The MHC II containing MPs could be susceptible to the shearing forces caused by centrifugation at 1400 x g. Following a 24 hour incubation of whole blood, however, PMNs isolated using the same centrifugation technique showed significant MHC II expression, although far less expression than PMNs isolated without using centrifugation (Figure 5.1). These observations are consistent with the hypothesis that there is a time-dependent integration of MP derived membrane and protein into the PMN membrane. These integrated proteins would then be resistant to removal by the centrifugal forces present during PMN isolation.

Further evidence that MPs might be attaching to the surface of PMNs was provided by the observation that significant levels of MHC II acquisition occurred when PMNs and PBMC were co-cultured at 4^oC. MPs are unable to efficiently fuse with PMN membranes at such a low temperature since plasma membrane fluidity is drastically reduced at this temperature [179,222,223]. We observed detectable levels of MHC Class II

molecules on PMNs within 40 minutes of co-culture at 4°C, however, it is likely that significant MHC II acquisition would have occurred sooner than 40 minutes. (Figure 5.2B). The minimal time for this experiment was defined by the time interval required to label cells for flow cytometric analysis. That is, PMNs were combined with PBMCs and monoclonal antibody labelling for MHC II was initiated immediately (time = 0min). Over the course of labelling with primary and secondary antibodies, PMNs were exposed to MHC II proteins expressed by other cell populations present in the wells. This experiment was carried out at 4°C since mammalian cell transcription and translation is markedly decreased at 4°C [215] thereby limiting endogenous gene expression. A parallel experiment performed at 37 °C showed no difference in the level of MHC II acquisition by PMNs (data not shown) which supports passive acquisition and argues against endogenous expression. These observations are also consistent with reports that spontaneous fusion of MPs and cells can occur at 4°C, mediated by SNARE proteins [179].

Another experiment involved the co-incubation of PMNs with PBMCs separated by a 0.4μm transwell membrane such that direct cell contact between PMNs and MHC II^+ cells was prevented (Figure 5.2B). No acquisition of MHC II by PMNs was detected by flow cytometry, but this result could have several interpretations. As MPs range in size from 0.05-1μm [153], perhaps some of the smaller MHC II expressing MPs were able to cross the transwell membrane and fuse with PMNs, but not in sufficient quantity to be detected by flow cytometry. It is also possible that many of the transwell pores were blocked by PBMCs which settled on the transwell membrane, thereby blocking MP access to the bottom compartment which contained PMNs. The adherent nature of MPs might also preclude them from reaching the bottom compartment as they might adhere to other PBMCs, MPs, or the transwell membrane itself. Alternatively, perhaps MHC II

proteins are only carried by larger MPs which were unable to cross the transwell membrane.

Direct evidence for bovine PMN passive acquisition versus endogenous expression of MHC II was provided by experiments which demonstrated the transfer of murine MHC II (Figure 5.3). Although this experiment didn't eliminate the possibility of endogenous MHC II expression, it conclusively supported our hypothesis that bovine PMNs could passively acquire protein, and demonstrated, at least using the murine model, that there was no species-specific limitation to this phenomenon.

Finally, we endeavoured to determine if bovine PMNs could be stimulated to express MHC class II on their surface, as had been reported with PMNs from other species [80,88,207,208]. Throughout our experiments with bovine PMNs, we tried to duplicate previously reported results such as the induction of MHC II expression following co-culture with certain cytokines (i.e. IFN-γ, GM-CSF, Figure 5.2A) or with a soluble T-cell factor. To examine the potential effect of soluble T-cell factors on MHC II expression in bovine PMNs, we co-cultured PMNs with activated T-cells which were separated by a 0.4μm transwell membrane (Figure 5.2B). Here, the transwell membrane facilitates an exchange of secreted soluble factors but prevents direct cell contact. None of these treatments induced detectable expression of MHC II on purified bovine PMNs,

The Role of Apoptosis and Necrosis in Passive Membrane/Protein Transfer

Following the observation that B-cells were the primary MHC II donor cell (Figure 5.4B) and a high percentage of B-cells underwent apoptosis within 24 hours of *in vitro* co-culture with PMNs (Figure 5.4A), the hypothesis was formulated that PMNs

could acquire membrane fragments and their associated integral membrane proteins from MPs shed from cells undergoing apoptosis. To confirm that donor cell apoptosis was important for the transfer of membrane proteins to PMNs, bovine PMNs were co-cultured with an ovine sheep B-cell line (Clone 2 B-cells). This cell line undergoes little spontaneous apoptosis during culture and no detectable MHC II was detected on PMNs co-cultured with Clone2 B-cells. To determine the efficiency of protein transfer using several different apoptosis-inducing techniques were attempted. Clone 2 B-cells were cultured with two different chemicals that induce apoptosis, Anisomycin (1μg/ml) and Doxorubicin hydrocholoride (1mM), or rendered apoptotic by γ-irradiation. However, it was difficult to determine if the induction of apoptosis in Clone 2 B-cells increased their capacity to be MHC II donors since flow cytometry could not distinguish between bovine PMNs and apoptotic Cl2 B cells. Therefore, Cl2 B cell membranes were obtained by using repeated freeze/thaws followed by a short sonication step to generate lysed cell membranes which were then co-cultured with PMNs (Figure 5.4B). PMNs passively acquired an equivalent level of MHC II from lysed Cl2 cell membranes and apoptotic bovine B-cells. The Cl2 cell transfer system was somewhat artificial but these observations raised the possibility that both apoptotic and necrotic cells could function as donors of integral membrane proteins.

Further evidence that a variety of proteins could be transferred to PMNs from apoptotic cells came from the observation that T-cells, gamma-irradiated to induce apoptosis, also transferred CD3 protein to PMNs (Figure 5.5). It is interesting to note, however, that the level of CD3 acquisition by PMNs was not equivalent to the level of MHC II acquisition. The reasons for this apparent difference were not critically addressed

but there are several possible explanations. There are relatively more MHC II^+ cells (B cells, monocytes, and some T-cells) than $CD3^+$ (T-cells) within purified PBMC populations and these cells may release a larger number of MPs than γ-irradiated T-cells. Alternatively, MPs might preferentially carry more MHC II protein than CD3 protein or the different methods of inducing cell death (i.e. γ-irradiation versus culture induced cell death) may alter the physical attributes of MPs and influence either their release from donor cells or attachment to PMNs.

In addition, biotin or PKH labelled bovine PBMCs were co-cultured with unlabeled PMNs for 24h, during which time nearly all PMNs acquired sufficient biotinylated proteins or PKH label on their surface to be detected with flow cytometry (Figure 5.6). Thus, although it was not possible to determine if this transfer was selective, this was evidence that both membrane fragments as well as multiple membrane proteins could be transferred to PMNs.

Model of Passive Acquisition

In Chapter 5, a theoretical model was designed which outlined a possible mechanism of membrane lipid and protein transfer to bovine PMNs (Figure 5.7). After the induction of apoptosis or necrosis, cells are known to release MPs which may or may not contain integral membrane proteins [131]. Such vesicles demonstrate increased adherent properties [216], and it was hypothesized that MPs adhered to PMNs and eventually became incorporated into the PMN plasma membrane. Based on this model, it is unlikely that proteins can insert into the PMN membrane in the improper orientation. This latter possibility was, however, included in the model. The detection of passively acquired biotinylated proteins on the surface of PMNs provides evidence that at least some

proteins were present in the correct orientation as SA-FITC is a large molecular complex which is excluded from viable cells and thus detection by flow cytometry was consistent with binding to biotinylated extra-cellular proteins.

Mechanism of Regulation of Membrane Transfer

The lack of variation in the amount of MHC II acquisition by PMNs, whether the MHC II source was from apoptotic, necrotic, xenogeneic, allogeneic or syngeneic cells, suggests that the mechanism of acquisition is not specific to one type of syngeneic cell, but is rather a more general process, perhaps inherent to the nature of MPs themselves. Since both apoptotic and necrotic cells can form MPs, these observations were consistent with the hypothesis. As described in Chapter 1, MPs have unique membrane characteristics which distinguish them from other cell membranes or debris. These unique properties may provide a specific signal which is important for the efficient acquisition of MP membranes and proteins by PMNs. It is also plausible that PMNs could acquire MPs shed from live cells. This investigation was limited to the use of apoptotic and necrotic cells since these systems provided rapid transfer of detectable levels of labelled proteins and membranes. Detection of this transfer by flow cytometry was clearly limited by the sensitivity of this methodology. All cells have an inherent level of autofluorescence and to detect a specific fluorescent signal it must exceed autofluorescence. Flow cytometry often requires 500-1000 fluorescent molecules on the surface of a cell to detect a specific signal [224]. Thus, the possibility cannot be disregarded that viable cells participate in MP-mediated transfer of membranes and proteins but at a level below the threshold of detection with flow cytometry.

Whatever the mechanism of passive membrane transfer, it is assumed that there are regulatory mechanisms to ensure both the specificity and the amplitude of this process. There is a growing body of literature regarding the diverse regulatory mechanisms which occur throughout cellular biology. Since the passive transfer of membranes and proteins between cells is a relatively new area of scientific exploration, these regulatory systems are largely unknown. Like all mammalian cells, PMNs possess numerous proteins involved in signalling pathways that control many aspects of cellular function, including gene transcription, cytokine-induced responses, phagocytosis, and cell motility. Many of these regulatory proteins are involved in the control of multiple intracellular signalling pathways and the functions of many proteins are not fully characterized. Therefore, it is probable that some of these proteins may play a role in regulating the passive acquisition of MPs and their associated proteins. For example, guanine exchange factors (GEF) of the Vav family are critical activators of Rho GTPases, which control actin cytoskeletal reorganization and gene transcription. Integrins have been characterized extensively as adhesion receptors capable of transducing signals inside the cell. Integrin-mediated adhesive interactions are known to regulate different selective cell responses, such as transmigration into the inflammatory site, cytokine secretion, production of reactive oxygen intermediates, degranulation and phagocytosis [225]. In fact, integrins are so involved in the regulation of neutrophil functioning that it would be surprising if they weren't key regulators in the acquisition of membrane proteins. They could act as primary adhesion molecules for protein-transporting microvesicles and subsequent membrane fusion, or they could be far more intricately involved in the downstream signalling processes. Their involvement in signal transduction, particularly that involving tyrosine kinase activity, has drawn significant

attention. Another likelihood is that newly acquired proteins only elicit their effects on recipient cells that have the proper cellular machinery or communication pathways to regulate these proteins. If microvesicles can fuse with many different cell types, they risk fusing with unintended recipients. Cells that acquire proteins which cannot access regulatory pathways may simply be endocytosed and degraded. There is, however, a possibility that newly acquired proteins can access downstream regulators as it has been shown that many distinct cell surface receptors can trigger responses that converge on common downstream intracellular pathways, such as the activation of caspases and transcription factors, or the control of apoptosis in PMNs [226]. That is, each signalling pathways may not be activated by a single receptor protein.

As described in Chapter 1, regulation of MP-mediated protein transfer could also occur at the level of the donor cell. For example, limited subsets of proteins might be incorporated into the MP or the generation of MPs may be regulated [153]. The controlled expression of SNARE proteins on both MPs and the recipient cell may play an important role in mediating membrane fusion [179]. The state of donor cell activation or stimulation can alter MP formation and release [131] and therefore extrinsic factors (cytokines, hormones, immunologic environment, etc.) might influence this process [138]. From the perspective of PMNs, the cytokine environment will likely affect its function and state of activation which may in turn impact its ability to acquire membrane proteins. Furthermore, because PMNs are known to be relatively short lived, there is an additional level of control by factors which may influence PMN lifespan (Chapter 3). Additionally, without the continual presence of MP shedding donor cells, any protein acquired by PMNs, at a site of inflammation for example, would eventually be lost by processes such as endocytosis or surface membrane turn-over. Finally, it is possible passively acquired

proteins simply function to decorate PMNs with adhesion molecules, receptors, or antigen presenting proteins that can elicit effects from the surface of a cell without any need for downstream regulation.

Direct Evidence for Passive Transfer Using Confocal Microscopy

In Chapter 6, we confirmed the transfer of both membrane fragments as well as surface proteins from PBMCs to PMNs using confocal microscopy. Confocal microscopy is a powerful tool used to visualize phenomena at the level of individual cells and to elucidate the mechanism by which cellular interactions may occur. One of the limitations of confocal microscopy, however, is that the fluorescent dyes used to label the different cell populations can bleach when exposed for a prolonged period of time (20-30 seconds) to the lasers that excite fluorescence. This means that there was limited time to view each individual interaction between PMNs and the microparticles (MPs) adhered to them before the fluorescent label was quenched. The short viewing time, coupled with the fact that the cells moved during the viewing process, made following the progression of events with any one particular cell very difficult. Although we were unable to capture the actual process of fusion between microparticle and PMN, we were able to provide direct evidence, from serial photographs, for MP attachment and fusion with PMN plasma membranes, as described in Chapter 6. A variety of membrane and membrane protein labelling experiments provided direct evidence for the formation and release of MPs from fluorescently labelled PBMCs and the subsequent attachment of MPs to the surface of PMNs (Figure 6.1). These experiments also provided direct evidence that fluorescently labelled membrane lipids and proteins incorporated and diffused throughout the recipient PMN plasma membrane (Figure 6.2 and 6.3). An alternative explanation, which is very

similar to the hypothesis described above, is simply that non-vesicular membrane fragments can adhere and fuse with PMN membranes. Perhaps both scenarios can occur. It would be interesting to develop a methodology whereby a particular PMN:MP interaction could be viewed from start to finish, and therefore capture the details of the fusion event. Perhaps the MP doesn't fuse completely with the PMN membrane, but rather deposits only a small membrane fragment within the PMN membrane. Many mechanistic questions remain unanswered, however the above data supports our previous hypotheses that the passive acquisition of membrane fragments and integral membrane proteins is mediated by MPs. These MPs initially adhere to the surface of the recipient cell and then there is a time-dependent integration and diffusion of acquired material into PMNs.

It was also curious to note that we didn't observe reciprocal membrane transfer from PMNs to PBMCs, despite the death of many PMNs during co-culture. This uni-directional transfer might support the idea that MP transfer involves a specific signal from the dying cells which enables PMNs to fuse with shed MPs, but this signal may not invoke the same response in PBMCs. The transfer of membrane proteins to T-cells, B-cells and NK cells has been shown to involve interactions in the immunological synapse (discussed in Chapter 1), and therefore a requirement for intimate cell-cell contact might preclude MP-mediated transfer of membranes and proteins from PMNs to PBMCs. If MP transfer is mediated by proteins involved in the phagocytic pathway, then we might expect at least some MP acquisition by monocytes. It is also possible that PMNs possess specific receptors which facilitate MP attachment and fusion, however, as all membranous cells have the capacity to express SNARE proteins [178], this possibility seems unlikely. Finally, our confocal photographs showed many microparticles in the media

that had been shed from the PBMC population, but no MPs were observed which contained the fluorescent stain used to label PMNs. Thus, it is possible that PMNs do not efficiently generate MPs or they were prevented from forming and shedding MPs by some as yet unknown signal.

Evidence for the Transfer of Functional Proteins to PMNs

In Chapter 6**,** we reported experiments which determined that passively acquired proteins remained functional and could alter PMN biology. Specifically, PMN acquisition of MHC class II from xenogeneic cells resulted in PMNs being able to stimulate proliferation and cytokine production by the xenoreactive bovine T-cells (Figure 6.6 and 6.7). This observation confirmed the possibility of passive transfer of functional protein to PMNs. The structural integrity of the transferred MHC II molecules and bound peptide must have been maintained despite Clone 2 B-cell treatment with freeze/thaw and sonication. Relatively less T-cell stimulation was observed, however, following PMN stimulation versus Clone2 B-cell stimulation. These diminished T-cell responses may be explained by some denaturation of MHC II molecules or peptide disassociation during Clone 2 B-cell lysis and the passive transfer of MHC II molecules to PMNs. Another reason that PMNs did not function as efficient APCs may be that substantially lower levels of surface MHC II was achieved on PMNs following passive transfer as compared to the MHC II expression by other professional APCs. A comparison of Clone 2 B-cells and PMNs, following passive acquisition of ovine MHC II revealed greater than 44-fold lower level of detectable MHC II on PMNs (Figure 6.6B). Therefore, the density of antigen available for T-cell stimulation may explain the reduction in T-cell cytokine expression and proliferative responses. Several steps were

taken throughout these experiments to assure that lysed Clone 2-B-cell membrane fragments (or MPs) were not the cause of T-cell stimulation. Following Clone 2 B-cell lysis, the lysate was centrifuged at 216 x g for 3 minutes to remove any intact cells and no cells were seen when the lysate was viewed using an inverted light microscope. Furthermore, following co-incubation of PMNs with Clone 2 B-cells lysates, the PMNs were washed 3 times with PBSA to remove unattached Clone 2 B-cell fragments. An aliquot of the supernatant from the final PMN wash was also added to each Clone 2-specific T-cell line but no detectable proliferative response was induced. Therefore, it was concluded that the induction of an MLR by PMNs, which had passively acquired ovine MHC II, was due to MHC II molecules attached to the surface of PMNs.

Neither the passive transfer to nor the endogenous expression of co-stimulatory molecules by PMNs was analyzed during the present investigation. The absence of co-stimulatory molecules on PMNs may be another explanation for the reduced capacity of PMNs to stimulate xenoreactive T-cell responses. There is a reduced requirement for co-stimulatory molecules during activation of primed T-cells but if PMNs lack appropriate co-signals this may still limit T-cell activation. A lack of cross-species recognition of co-stimulatory molecules does not appear to have limited T-cell responses since ovine B-cells functioned as effective inducers of xenoreactive bovine T-cells. Thus, further analysis of co-stimulatory molecule expression on Clone 2 B-cells and the transfer of these molecules to bovine PMNs may provide further insight into the capacity of PMNs to function as APCs.

It would also be worthwhile to determine if the level of PMN antigen presentation would be significantly altered if membrane proteins were passively acquired from apoptotic versus necrotic cells. There is evidence that an interaction between a

phagocyte (macrophage) and either apoptotic or necrotic cells can significantly alter the function of the recipient cell. Macrophages that phagocytosed apoptotic cell bodies had a reduced capacity to function as APCs when compared to cells that had phagocytosed necrotic cell bodies [83]. It would be interesting to determine if a similar modulation of PMN function could occur. This may have significant implications for PMN function at sites of inflammation. For example, if PMNs acquired membrane proteins from apoptotic cells rather than necrotic cells then there may be no T-cell stimulation but rather the induction of anergy or immunological quiescence.

Finally, if bovine PMNs can acquire intact and functional MHC II proteins at sites of inflammation then this may have significant implications for the transport of immunological information to the draining lymph nodes. There is evidence that soluble antigens may not be able to efficiently enter into the cortex of lymph nodes [135] where T-cell interactions with interdigitating DCs first occur. Thus, PMNs may provide an alternative antigen delivery system by carrying passively acquired MHC II proteins into the lymph node. It may not be necessary for PMNs to function directly as APCs since it has been reported that PMNs can 'regurgitate' processed peptide antigens, which can then be presented by other APCs [74]. Potter *et. al.* showed that PMNs, which expressed MHC I and had phagocytosed and processed antigen, were able to stimulate antigen primed T-cells. They were also able to regurgitate antigen into the surrounding milieu, which could in turn bind to MHC I molecules on the surface of the same cell or to neighbouring cells. Specifically, MHC-mismatched PMNs and fixed, syngeneic macrophages or DCs were co-incubated together with an antigen specific T-cell line. The PMNs lacked expression of K^b and were therefore unable to present antigen to T-cells, even when they were genetically compatible. As PMNs were only able to process and

not present antigen, and fixed APCs were unable to process but could present antigen, it was concluded that PMNs had somehow transferred antigen to the macrophages or DCs for presentation. It was postulated that this transfer may have occurred through deposition of antigenic peptide in the groove of empty MHC molecules on the surface of macrophages or DCs. The finding that MHC molecules are less stable without bound peptide was not addressed in this particular study. This study lacked, however, a comparison of T-cell stimulation by fixed APCs which had acquired regurgitated antigen versus fixed APCs which had processed antigen already bound to their MHC I prior to fixation. This comparison would have determined the relative efficiency of passive versus active acquisition for antigen presentation to CD8 T-cell. Nonetheless, the above study along with the knowledge that DCs and B-cells can physically remove surface antigen or integral membrane proteins from cells [134,136,137] provide a basis for the conjecture that PMN passive acquisition of membrane proteins *in vivo* may facilitate the dissemination of antigenic and other protein-related information. Furthermore, since PMNs can phagocytose and degrade a wide range of biological material then they may play an important role in the preparation of antigenic peptides for transfer to professional APCs as alluded to by Potter *et. al.* [74].

Finally, PMNs which have acquired MHC II loaded with peptide may have the capacity to form cognate interactions with TCRs on CD4 T-cells. As previously discussed, this protein endows them with the ability to either stimulate T-cells or render them tolerogenic, depending on PMN expression of appropriate co-signalling proteins and the state of T-cell activation. If PMNs passively acquire co-receptor proteins, such as CD80 and CD86, part of their role might then be the amplification of an existing immune response or even the primary activation of antigen-specific T-cells. Alternatively, if

either the antigen signal or co-signalling on PMNs was too weak, then this stimulation might result in the down-regulation of an existing T-cell response. A capacity to down-regulate or anergize T-cell responses might then imply that PMNs could play a role in the elimination of self-reactive T-cells at sites of inflammation following acquisition of MHC II containing self-peptides. Considering the large numbers of PMNs which can be recruited to sites of inflammation, their cumulative effect on an immune response may be very significant. A next step in determining the biological significance of PMN passive acquisition of membrane proteins may be to extend this research to include *in vivo* experimentation. *Ex vivo* loading of PMNs with MHC II (or other proteins) and injecting these cells into sites of inflammation, the blood or into afferent lymphatics might provide a system to determine if PMNs can induce either antigen-specific T cell activation or tolerance.

Further evidence that PMNs could passively acquire functional membrane proteins was provided by the experiments with membrane fragments and protein from a BAdV-3 permissive cell population (293 cells). This membrane transfer resulted in enhanced BAV304 infection of PMNs (Figure 6.5). The interpretation of this observation was limited, however, by an assumption that 293 membrane transfer resulted in PMN acquisition of a BAdV-3-specific receptor protein. Direct confirmation that increased viral infection was due to the passive transfer of a viral receptor protein would require confirmation that a specific receptor protein was transferred along with antibody blocking experiments to demonstrate that this protein was required for increased infection by BAV304. Previous investigators have clearly shown that the passive transfer of known viral receptor proteins can alter viral tropism [145,154]. These previous observations are consistent with the current evidence that passive acquisition of 293 cell membranes

increased BAdV-3 infection of PMNs. Furthermore, these observations are important since they suggest that passive acquisition of functional membrane proteins may have biological implications beyond alteration of immune responses. The significance of passive acquisition of viral receptor protein, however, may also be limited by a number of other cellular functions. There are several steps involved in virus penetration and replication beyond the primary limiting step of viral attachment. For a productive viral infection to occur, the virus must also be able to penetrate the host cell membrane and then effectively use the host cell machinery for replication. Thus, no biological effect may be observed if passive acquisition of viral receptor proteins is not be coupled with a cell's competence to support viral replication. This issue was not addressed in the present investigation since bovine PMNs did not support BAdV-3 replication and viral infection was monitored through the expression of the GFP reporter gene.

A Potential for Cytoplasmic Protein and mRNA Transfer

An additional observation worthy of further discussion was the passive acquisition of cytoplasmic material by PMNs. The ovine B cell line expressing cytoplasmic GFP (Cl2-GFP) enabled photography of the formation and release of GFP containing MPs from γ-irradiated Clone 2-GFP B-cells (Figure 6.4). These GFP labelled MPs could then be visualized with confocal microscopy and these analyses revealed that GFP had been transferred to the cytoplasm of PMNs. Due to rapid photo-bleaching of GFP fluorescence it was difficult to visualize the transfer of small amounts of GFP from MPs to PMNs. Thus the initial fluorescence signal within the recipient PMN was limited and the signal was rapidly quenched by photo-bleaching. However, cytoplasmic protein transfer was observed and this raises the possibility that mRNA was also transferred. It is

logical that if MPs fuse with the plasma membrane, then the cytoplasmic contents of MPs may also be transferred to the recipient cell. Endonucleases within apoptotic cells may degrade much of the mRNA being produced but the possibility remains that intact mRNA may be packaged within MPs or that MPs containing mRNA could be shed from live cells. Thus, MPs may also function as a transfer vehicle for mRNA which could then be translated into protein within the recipient cell. This passive transfer of mRNA might be one possible explanation for previous reports that MHC class II mRNA was detected in human PMNs [88]. It remains to be determined, however, if sufficient mRNA could be transferred to alter the biology of the recipient cell.

Summary and Conclusions

The mammalian immune system, exquisitely designed to defend the host against a variety of pathogens, must be able to keep pace with pathogens as they evolve. Pathogens have an incredible ability to rapidly adapt and evolve to assure their survival and thus, the immune system must utilize a variety of mechanisms to control infection and limited disease. Effective communication among leukocytes is of utmost importance to ensure a coordinated and integrated immune response. Membrane protein transfer from apoptotic or necrotic cells to viable cells offers another vehicle by which cells may communicate, even after cell death. Further investigations may confirm that PMNs can present antigen directly to T-cells or perhaps PMNs merely function as a vehicle for antigen trafficking to other profession APCs. Whatever the outcome of future investigations, the present investigation provides evidence that the passive acquisition of proteins by PMNs has the potential to alter important immunological functions. In addition, this work has substantial implications for investigators when characterizing

proteins present on the surface of cells. The presence of a specific protein on the surface of a cell cannot be interpreted as direct evidence that the protein was expressed by that cell. Furthermore, evidence that a protein was passively acquired by a cell does not mean that the protein will remain inert, but might play an important role in the biology of that cell. Thus, the current investigation revealed that a cell's biological potential may not be limited by the repertoire of genes and proteins expressed within the cell.

CHAPTER 8

REFERENCES

1. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect. 2003;5:1317-1327.

2. Grewal AS, Rouse BT, Babiuk LA. Mechanisms of recovery from viral infections: destruction of infected cells by neutrophils and complement. J Immunol. 1980;124:312-319.

3. Rouse BT, Babiuk LA, Henson PM. Neutrophils in antiviral immunity: inhibition of virus replication by a mediator produced by bovine neutrophils. J Infect Dis. 1980;141:223-232.

4. Baggiolini M, Dewald B. The neutrophil. Int Arch Allergy Appl Immunol. 1985;76 Suppl 1:13-20.

5. Maianski NA, Maianski AN, Kuijpers TW, Roos D. Apoptosis of neutrophils. Acta Haematol. 2004;111:56-66.

6. Cline M. The White Cell. Cambridge, MA: Harvard University Press; 1975.

7. Bertram TA. Neutrophilic leukocyte structure and function in domestic animals. Adv Vet Sci Comp Med. 1985;30:91-129.

8. Peyron P, Bordier C, N'Diaye EN, Maridonneau-Parini I. Nonopsonic phagocytosis of Mycobacterium kansasii by human neutrophils depends on cholesterol and is mediated by CR3 associated with glycosylphosphatidylinositol-anchored proteins. J Immunol. 2000;165:5186-5191.

9. Paape MJ, Bannerman DD, Zhao X, Lee JW. The bovine neutrophil: Structure and function in blood and milk. Vet Res. 2003;34:597-627.

10. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood. 2003;102:2660-2669.

11. Francois S, El Benna J, Dang PM, Pedruzzi E, Gougerot-Pocidalo MA, Elbim C. Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad. J Immunol. 2005;174:3633-3642.

12. Sengelov H. Complement receptors in neutrophils. Crit Rev Immunol. 1995;15:107-131.

13. Murphy P. The Neutrophil. New York, NY: Plenum Publishing Corporation; 1976.

14. Kunkel SL, Lukacs N, Strieter RM. Expression and biology of neutrophil and endothelial cell-derived chemokines. Semin Cell Biol. 1995;6:327-336.

15. Woodman RC, Johnston B, Hickey MJ, et al. The functional paradox of CD43 in leukocyte recruitment: a study using CD43-deficient mice. J Exp Med. 1998;188:2181-2186.

16. Germain RN, Margulies DH. The biochemistry and cell biology of antigen processing and presentation. Annu Rev Immunol. 1993;11:403-450.

17. Janeway CAJ, Walport, M. & Shlomchik, M.J. Immunobiology (ed 5th Edition). New York: Garland Publishing; 2001.

18. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.

19. Heath WR, Belz GT, Behrens GM, et al. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol Rev. 2004;199:9-26.

20. Pardoll DM. Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol. 2002;2:227-238.

21. Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science. 2002;296:1873-1876.

22. Sadasivan B, Lehner PJ, Ortmann B, Spies T, Cresswell P. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity. 1996;5:103-114.

23. Rajagopal D, Bal V, George A, Rath S. Diversity & overlap in the mechanisms of processing protein antigens for presentation to T cells. Indian J Med Res. 2004;120:75-85.

24. Janeway C, Travers, P. Immuno Biology (ed 3rd). New York: Garland Publishing Inc.; 1997.

25. Campbell DJ, Serwold T, Shastri N. Bacterial proteins can be processed by macrophages in a transporter associated with antigen processing-independent, cysteine protease-dependent manner for presentation by MHC class I molecules. J Immunol. 2000;164:168-175.

26. Sigal LJ, Rock KL. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. J Exp Med. 2000;192:1143-1150.

27. Desjardins M. ER-mediated phagocytosis: a new membrane for new functions. Nat Rev Immunol. 2003;3:280-291.

28. Gromme M, Uytdehaag FG, Janssen H, et al. Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci U S A. 1999;96:10326-10331.

29. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol. 1997;15:821-850.

30. Pinet V, Vergelli M, Martin R, Bakke O, Long EO. Antigen presentation mediated by recycling of surface HLA-DR molecules. Nature. 1995;375:603-606.

31. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. J Exp Med. 2000;191:1513-1524.

32. Mukherjee P, Dani A, Bhatia S, et al. Efficient presentation of both cytosolic and endogenous transmembrane protein antigens on MHC class II is dependent on cytoplasmic proteolysis. J Immunol. 2001;167:2632-2641.

33. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med. 1976;143:1283-1288.

34. Bevan MJ. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. J Immunol. 1976;117:2233-2238.

35. Gooding LR, Edwards CB. H-2 antigen requirements in the in vitro induction of SV40-specific cytotoxic T lymphocytes. J Immunol. 1980;124:1258-1262.

36. den Haan JM, Bevan MJ. Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. J Exp Med. 2002;196:817-827.

37. den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med. 2000;192:1685-1696.

38. Pooley JL, Heath WR, Shortman K. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol. 2001;166:5327-5330.

39. Ke Y, Kapp JA. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. J Exp Med. 1996;184:1179-1184.

40. Limmer A, Ohl J, Kurts C, et al. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. Nat Med. 2000;6:1348-1354.

41. Ramirez MC, Sigal LJ. Macrophages and dendritic cells use the cytosolic pathway to rapidly cross-present antigen from live, vaccinia-infected cells. J Immunol. 2002;169:6733-6742.

42. Rock KL, Rothstein L, Gamble S, Fleischacker C. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. J Immunol. 1993;150:438-446.

43. Carbone FR, Bevan MJ. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. J Exp Med. 1990;171:377-387.

44. Houde M, Bertholet S, Gagnon E, et al. Phagosomes are competent organelles for antigen cross-presentation. Nature. 2003;425:402-406.

45. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86-89.

46. Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J Immunol. 2001;166:3717-3723.

47. Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol. 2002;2:185-194.

48. Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001;7:297-303.

49. Ferguson TA, Herndon J, Elzey B, Griffith TS, Schoenberger S, Green DR. Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. J Immunol. 2002;168:5589-5595.

50. Albert ML, Pearce SF, Francisco LM, et al. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med. 1998;188:1359-1368.

51. Kovacsovics-Bankowski M, Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science. 1995;267:243-246.

52. Guermonprez P, Saveanu L, Kleijmeer M, Davoust J, Van Endert P, Amigorena S. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature. 2003;425:397-402.

53. Gagnon E, Duclos S, Rondeau C, et al. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell. 2002;110:119-131.

54. Ramachandra L, Song R, Harding CV. Phagosomes are fully competent antigen-processing organelles that mediate the formation of peptide:class II MHC complexes. J Immunol. 1999;162:3263-3272.

55. Hurwitz AA, Yu TF, Leach DR, Allison JP. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. Proc Natl Acad Sci U S A. 1998;95:10067-10071.

56. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med. 1999;190:355-366.

57. Latouche JB, Sadelain M. Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells. Nat Biotechnol. 2000;18:405-409.

58. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol. 2003;3:269-279.

59. Bhardwaj N, Seder RA, Reddy A, Feldman MV. IL-12 in conjunction with dendritic cells enhances antiviral CD8+ CTL responses in vitro. J Clin Invest. 1996;98:715-722.

60. Ahn HJ, Maruo S, Tomura M, et al. A mechanism underlying synergy between IL-12 and IFN-gamma-inducing factor in enhanced production of IFN-gamma. J Immunol. 1997;159:2125-2131.

61. Bromley SK, Burack WR, Johnson KG, et al. The immunological synapse. Annu Rev Immunol. 2001;19:375-396.

62. Fanger NA, Liu C, Guyre PM, et al. Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. Blood. 1997;89:4128-4135.

63. Johnson HM, Russell JK, Pontzer CH. Staphylococcal enterotoxin superantigens. Proc Soc Exp Biol Med. 1991;198:765-771.

64. McKay DM. Bacterial superantigens: provocateurs of gut dysfunction and inflammation? Trends Immunol. 2001;22:497-501.

65. Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. J Immunol. 1995;155:5249-5256.

66. McDevitt HO. Discovering the role of the major histocompatibility complex in the immune response. Annu Rev Immunol. 2000;18:1-17.

67. Haller GW, Lima B, Kunisaki SM, et al. MHC alloantigens elicit secondary, but not primary, indirect in vitro proliferative responses. J Immunol. 2002;169:3613-3621.

68. Shishido S, Naziruddin B, Xu XC, Howard T, Mohanakumar T. Indirect recognition of porcine xenoantigens by human CD4+ T cell clones. Transplantation. 1998;65:706-712.

69. Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv Immunol. 1979;27:51-177.

70. van Spriel AB, van den Herik-Oudijk IE, van Sorge NM, Vile HA, van Strijp JA, van de Winkel JG. Effective phagocytosis and killing of Candida albicans via targeting FcgammaRI (CD64) or FcalphaRI (CD89) on neutrophils. J Infect Dis. 1999;179:661-669.

71. Chang KP. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. Am J Trop Med Hyg. 1981;30:322-333.

72. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335-376.

73. Van den Steen PE, Proost P, Grillet B, et al. Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational

modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis. Faseb J. 2002;16:379-389.

74. Potter NS, Harding CV. Neutrophils process exogenous bacteria via an alternate class I MHC processing pathway for presentation of peptides to T lymphocytes. J Immunol. 2001;167:2538-2546.

75. Unanue ER. Antigen Presenting Cells: Encyclopedia of Immunology. New York, NY: Academic; 1992.

76. Mannie MD, Nardella JP, White GA, Arnold PY, Davidian DK. Class II MHC/peptide complexes on T cell antigen-presenting cells: agonistic antigen recognition inhibits subsequent antigen presentation. Cell Immunol. 1998;186:111-120.

77. Okuda K, Neely BC, David CS. Expression of H-2 and Ia antigens on mouse peritoneal neutrophils. Transplantation. 1979;28:354-356.

78. Mudzinski SP, Christian TP, Guo TL, Cirenza E, Hazlett KR, Gosselin EJ. Expression of HLA-DR (major histocompatibility complex class II) on neutrophils from patients treated with granulocyte-macrophage colony- stimulating factor for mobilization of stem cells. Blood. 1995;86:2452-2453.

79. Reinisch W, Tillinger W, Lichtenberger C, et al. In vivo induction of HLA-DR on human neutrophils in patients treated with interferon-gamma. Blood. 1996;87:3068.

80. Vachiery N, Totte P, Balcer V, Martinez D, Bensaid A. Effect of isolation techniques, in vitro culture and IFNgamma treatment on the constitutive expression of MHC Class I and Class II molecules on goat neutrophils. Vet Immunol Immunopathol. 1999;70:19-32.

81. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3. J Immunol. 1993;151:1482-1490.

82. Smith WB, Guida L, Sun Q, et al. Neutrophils activated by granulocyte-macrophage colony-stimulating factor express receptors for interleukin-3 which mediate class II expression. Blood. 1995;86:3938-3944.

83. Barker RN, Erwig LP, Hill KS, Devine A, Pearce WP, Rees AJ. Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. Clin Exp Immunol. 2002;127:220-225.

84. Schwab R, Crow MK, Russo C, Weksler ME. Requirements for T cell activation by OKT3 monoclonal antibody: role of modulation of T3 molecules and interleukin 1. J Immunol. 1985;135:1714-1718.

85. Lanier LL, O'Fallon S, Somoza C, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J Immunol. 1995;154:97-105.

86. Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to antigen. Annu Rev Immunol. 1993;11:191-212.

87. Damle NK, Klussman K, Leytze G, Linsley PS. Proliferation of human T lymphocytes induced with superantigens is not dependent on costimulation by the CD28 counter-receptor B7. J Immunol. 1993;150:726-735.

88. Radsak M, Iking-Konert C, Stegmaier S, Andrassy K, Hansch GM. Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation. Immunology. 2000;101:521-530.

89. Iking-Konert C, Vogt S, Radsak M, Wagner C, Hansch GM, Andrassy K. Polymorphonuclear neutrophils in Wegener's granulomatosis acquire characteristics of antigen presenting cells. Kidney Int. 2001;60:2247-2262.

90. Fischer H, Gjorloff A, Hedlund G, et al. Stimulation of human naive and memory T helper cells with bacterial superantigen. Naive CD4+45RA+ T cells require a costimulatory signal mediated through the LFA-1/ICAM-1 pathway. J Immunol. 1992;148:1993-1998.

91. Windhagen A, Maniak S, Gebert A, Ferger I, Wurster U, Heidenreich F. Human polymorphonuclear neutrophils express a B7-1-like molecule. J Leukoc Biol. 1999;66:945-952.

92. Lloyd AR, Oppenheim JJ. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immunol Today. 1992;13:169-172.

93. Jack RM, Fearon DT. Selective synthesis of mRNA and proteins by human peripheral blood neutrophils. J Immunol. 1988;140:4286-4293.

94. Neuman E, Huleatt JW, Jack RM. Granulocyte-macrophage colony-stimulating factor increases synthesis and expression of CR1 and CR3 by human peripheral blood neutrophils. J Immunol. 1990;145:3325-3332.

95. Lord PC, Wilmoth LM, Mizel SB, McCall CE. Expression of interleukin-1 alpha and beta genes by human blood polymorphonuclear leukocytes. J Clin Invest. 1991;87:1312-1321.

96. Cicco NA, Lindemann A, Content J, et al. Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. Blood. 1990;75:2049-2052.

97. Lopez AF, Williamson DJ, Gamble JR, et al. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. J Clin Invest. 1986;78:1220-1228.

98. Dubravec DB, Spriggs DR, Mannick JA, Rodrick ML. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. Proc Natl Acad Sci U S A. 1990;87:6758-6761.

99. Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA. The neutrophil as a cellular source of chemokines. Immunol Rev. 2000;177:195-203.

100. Chertov O, Yang D, Howard OM, Oppenheim JJ. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. Immunol Rev. 2000;177:68-78.

101. Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. Adv Immunol. 1999;73:369-509.

102. Lefkowitz DL, Lefkowitz SS. Macrophage-neutrophil interaction: a paradigm for chronic inflammation revisited. Immunol Cell Biol. 2001;79:502-506.

103. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood. 1992;80:2012-2020.

104. Begley CG, Lopez AF, Nicola NA, et al. Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in vitro: a rapid and sensitive microassay for colony-stimulating factors. Blood. 1986;68:162-166.

105. Oehler L, Majdic O, Pickl WF, et al. Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics. J Exp Med. 1998;187:1019-1028.

106. Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. Annu Rev Immunol. 1992;10:267-293.

107. Benedict CA, Norris PS, Ware CF. To kill or be killed: viral evasion of apoptosis. Nat Immunol. 2002;3:1013-1018.

108. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol. 2002;2:965-975.

109. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature. 2000;407:784-788.

110. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26:239-257.

111. Kuby J. Immunology (ed 3rd Edition). New York: W.H. Freeman and Company; 1997.

112. Jesenberger V, Jentsch S. Deadly encounter: ubiquitin meets apoptosis. Nat Rev Mol Cell Biol. 2002;3:112-121.

113. Meier P, Finch A, Evan G. Apoptosis in development. Nature. 2000;407:796-801.

114. Takizawa T, Fukuda R, Miyawaki T, Ohashi K, Nakanishi Y. Activation of the apoptotic Fas antigen-encoding gene upon influenza virus infection involving spontaneously produced beta-interferon. Virology. 1995;209:288-296.

115. Cuconati A, White E. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev. 2002;16:2465-2478.

116. Everett H, McFadden G. Apoptosis: an innate immune response to virus infection. Trends Microbiol. 1999;7:160-165.

117. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol. 1980;68:251-306.

118. Andersson U, Wang H, Palmblad K, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med. 2000;192:565-570.

119. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999;285:248-251.

120. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002;418:191-195.

121. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood. 1997;89:1121-1132.

122. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000;405:85-90.

123. Koyama AH, Adachi A, Irie H. Physiological significance of apoptosis during animal virus infection. Int Rev Immunol. 2003;22:341-359.

124. Koyama AH, Irie H, Ueno F, Ogawa M, Nomoto A, Adachi A. Suppression of apoptotic and necrotic cell death by poliovirus. J Gen Virol. 2001;82:2965-2972.

125. Wajant H, Pfizenmaier K, Scheurich P. Non-apoptotic Fas signaling. Cytokine Growth Factor Rev. 2003;14:53-66.

126. Koyama AH, Arakawa T, Adachi A. Acceleration of virus-induced apoptosis by tumor necrosis factor. FEBS Lett. 1998;426:179-182.

127. Koyama AH, Fukumori T, Fujita M, Irie H, Adachi A. Physiological significance of apoptosis in animal virus infection. Microbes Infect. 2000;2:1111-1117.

128. Reddy SM, Hsiao KH, Abernethy VE, et al. Phagocytosis of apoptotic cells by macrophages induces novel signaling events leading to cytokine-independent survival

and inhibition of proliferation: activation of Akt and inhibition of extracellular signal-regulated kinases 1 and 2. J Immunol. 2002;169:702-713.

129. Evan G, Littlewood T. A matter of life and cell death. Science. 1998;281:1317-1322.

130. Sester DP, Beasley SJ, Sweet MJ, et al. Bacterial/CpG DNA down-modulates colony stimulating factor-1 receptor surface expression on murine bone marrow-derived macrophages with concomitant growth arrest and factor-independent survival. J Immunol. 1999;163:6541-6550.

131. Freyssinet JM. Cellular microparticles: what are they bad or good for? J Thromb Haemost. 2003;1:1655-1662.

132. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol. 1995;146:3-15.

133. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature. 1997;390:350-351.

134. Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J Immunol. 2001;166:3717-3723.

135. Gretz JE, Norbury CC, Anderson AO, Proudfoot AE, Shaw S. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. J Exp Med. 2000;192:1425-1440.

136. Dustin ML, Dustin LB. The immunological relay race: B cells take antigen by synapse. Nat Immunol. 2001;2:480-482.

137. Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. Nature. 2001;411:489-494.

138. Patel DM, Mannie MD. Intercellular exchange of class II major histocompatibility complex/peptide complexes is a conserved process that requires activation of T cells but is constitutive in other types of antigen presenting cell. Cell Immunol. 2001;214:165-172.

139. Patel DM, Dudek RW, Mannie MD. Intercellular exchange of class II MHC complexes: ultrastructural localization and functional presentation of adsorbed I-A/peptide complexes. Cell Immunol. 2001;214:21-34.

140. Patel DM, Arnold PY, White GA, Nardella JP, Mannie MD. Class II MHC/peptide complexes are released from APC and are acquired by T cell responders during specific antigen recognition. J Immunol. 1999;163:5201-5210.

141. Huang JF, Yang Y, Sepulveda H, et al. TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. Science. 1999;286:952-954.

142. Arnold PY, Davidian DK, Mannie MD. Antigen presentation by T cells: T cell receptor ligation promotes antigen acquisition from professional antigen-presenting cells. Eur J Immunol. 1997;27:3198-3205.

143. Carlin LM, Eleme K, McCann FE, Davis DM. Intercellular transfer and supramolecular organization of human leukocyte antigen C at inhibitory natural killer cell immune synapses. J Exp Med. 2001;194:1507-1517.

144. Tabiasco J, Espinosa E, Hudrisier D, Joly E, Fournie JJ, Vercellone A. Active trans-synaptic capture of membrane fragments by natural killer cells. Eur J Immunol. 2002;32:1502-1508.

145. Tabiasco J, Vercellone A, Meggetto F, Hudrisier D, Brousset P, Fournie JJ. Acquisition of viral receptor by NK cells through immunological synapse. J Immunol. 2003;170:5993-5998.

146. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity. 2001;15:751-761.

147. Poupot M, Fournie JJ. Spontaneous membrane transfer through homotypic synapses between lymphoma cells. Journal of Immunology. 2003;171:2517-2523.

148. Tabibzadeh SS, Kong QF, Kapur S. Passive acquisition of leukocyte proteins is associated with changes in phosphorylation of cellular proteins and cell-cell adhesion properties. Am J Pathol. 1994;145:930-940.

149. Devaux PF. Static and dynamic lipid asymmetry in cell membranes. Biochemistry. 1991;30:1163-1173.

150. Mann KG. Biochemistry and physiology of blood coagulation. Thromb Haemost. 1999;82:165-174.

151. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002;417:182-187.

152. Aupeix K, Hugel B, Martin T, et al. The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection. J Clin Invest. 1997;99:1546-1554.

153. Satta N, Toti F, Feugeas O, et al. Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. J Immunol. 1994;153:3245-3255.

154. Mack M, Kleinschmidt A, Bruhl H, et al. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med. 2000;6:769-775.

155. Barry OP, Pratico D, Lawson JA, FitzGerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. J Clin Invest. 1997;99:2118-2127.

156. Levene RB, Rabellino EM. Platelet glycoproteins IIb and IIIa associated with blood monocytes are derived from platelets. Blood. 1986;67:207-213.

157. Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. Blood. 2000;95:1317-1323.

158. Bergsmedh A, Szeles A, Henriksson M, et al. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A. 2001;98:6407-6411.

159. Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. Nature. 2000;405:299-304.

160. Lake JA, Jain R, Rivera MC. Mix and match in the tree of life. Science. 1999;283:2027-2028.

161. Holmgren L, Szeles A, Rajnavolgyi E, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood. 1999;93:3956-3963.

162. Spetz AL, Patterson BK, Lore K, Andersson J, Holmgren L. Functional gene transfer of HIV DNA by an HIV receptor-independent mechanism. J Immunol. 1999;163:736-742.

163. de la Taille A, Chen MW, Burchardt M, Chopin DK, Buttyan R. Apoptotic conversion: evidence for exchange of genetic information between prostate cancer cells mediated by apoptosis. Cancer Res. 1999;59:5461-5463.

164. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002;2:569-579.

165. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998;4:594-600.

166. Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996;183:1161-1172.

167. Thery C, Boussac M, Veron P, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol. 2001;166:7309-7318.

168. Blanchard N, Lankar D, Faure F, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol. 2002;168:3235-3241.

169. Vincent-Schneider H, Stumptner-Cuvelette P, Lankar D, et al. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. Int Immunol. 2002;14:713-722.

170. Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I, Telemo E. "Tolerosomes" are produced by intestinal epithelial cells. Eur J Immunol. 2001;31:2892-2900.

171. Skokos D, Le Panse S, Villa I, et al. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. J Immunol. 2001;166:868-876.

172. Dukers DF, Meij P, Vervoort MB, et al. Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. J Immunol. 2000;165:663-670.

173. Denzer K, van Eijk M, Kleijmeer MJ, Jakobson E, de Groot C, Geuze HJ. Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. J Immunol. 2000;165:1259-1265.

174. Gray D, Kosco M, Stockinger B. Novel pathways of antigen presentation for the maintenance of memory. Int Immunol. 1991;3:141-148.

175. Jahn R, Lang T, Sudhof TC. Membrane fusion. Cell. 2003;112:519-533.

176. Blott EJ, Griffiths GM. Secretory lysosomes. Nat Rev Mol Cell Biol. 2002;3:122-131.

177. Pelham HR. SNAREs and the specificity of membrane fusion. Trends Cell Biol. 2001;11:99-101.

178. Chen EH, Olson EN. Unveiling the mechanisms of cell-cell fusion. Science. 2005;308:369-373.

179. Weber T, Zemelman BV, McNew JA, et al. SNAREpins: minimal machinery for membrane fusion. Cell. 1998;92:759-772.

180. Rothman JE, Sollner TH. Throttles and dampers: controlling the engine of membrane fusion. Science. 1997;276:1212-1213.

181. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. Cell. 1997;89:263-273.

182. Lu M, Blacklow SC, Kim PS. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat Struct Biol. 1995;2:1075-1082.

183. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. Nature. 1997;387:426-430.

184. Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell. 1997;90:523-535.

185. Lin RC, Scheller RH. Structural organization of the synaptic exocytosis core complex. Neuron. 1997;19:1087-1094.

186. Savill JS, Henson PM, Haslett C. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. J Clin Invest. 1989;84:1518-1527.

187. Smith JB, McIntosh GH, Morris B. The migration of cells through chronically inflamed tissues. J Pathol. 1970;100:21-29.

188. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. J Exp Med. 1976;143:870-888.

189. Kerlin RL, Watson DL. The secondary immune response to Staphylococcus aureus vaccines in efferent popliteal lymph of sheep. Immunology. 1987;60:295-301.

190. Steer HW. Study of acute localised inflammation of the gastrointestinal tract: the effluent lymph. Gut. 1981;22:827-835.

191. Cowburn AS, Cadwallader KA, Reed BJ, Farahi N, Chilvers ER. Role of PI3-kinase-dependent Bad phosphorylation and altered transcription in cytokine-mediated neutrophil survival. Blood. 2002;100:2607-2616.

192. Ohmann HB, Campos M, McDougall L, Lawman MJ, Babiuk LA. Expression of tumor necrosis factor-alpha receptors on bovine macrophages, lymphocytes and polymorphonuclear leukocytes, internalization of receptor-bound ligand, and some functional effects. Lymphokine Res. 1990;9:43-58.

193. Kapp A, Zeck-Kapp G. Activation of the oxidative metabolism in human polymorphonuclear neutrophilic granulocytes: the role of immuno-modulating cytokines. J Invest Dermatol. 1990;95:94S-99S.

194. Nichani AK, Mena A, Popowych Y, et al. In vivo immunostimulatory effects of CpG oligodeoxynucleotide in cattle and sheep. Vet Immunol Immunopathol. 2004;98:17-29.

195. Sallerfors B. Endogenous production and peripheral blood levels of granulocyte-macrophage (GM-) and granulocyte (G-) colony-stimulating factors. Leuk Lymphoma. 1994;13:235-247.

196. Kawano Y, Takaue Y, Saito S, et al. Granulocyte colony-stimulating factor (CSF), macrophage-CSF, granulocyte-macrophage CSF, interleukin-3, and interleukin-6 levels in sera from children undergoing blood stem cell autografts. Blood. 1993;81:856-860.

197. Martino G, Grohovaz F, Brambilla E, et al. Proinflammatory cytokines regulate antigen-independent T-cell activation by two separate calcium-signaling pathways in multiple sclerosis patients. Ann Neurol. 1998;43:340-349.

198. Schwartzberg LS, Petak I, Stewart C, et al. Modulation of the Fas signaling pathway by IFN-gamma in therapy of colon cancer: phase I trial and correlative studies of IFN-gamma, 5-fluorouracil, and leucovorin. Clin Cancer Res. 2002;8:2488-2498.

199. Raggo C, Habermehl M, Babiuk LA, Griebel P. The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-gamma. J Gen Virol. 2000;81:2665-2673.

200. Chello M, Mastroroberto P, Quirino A, et al. Inhibition of neutrophil apoptosis after coronary bypass operation with cardiopulmonary bypass. Ann Thorac Surg. 2002;73:123-129; discussion 129-130.

201. Kettritz R, Xu YX, Kerren T, et al. Extracellular matrix regulates apoptosis in human neutrophils. Kidney Int. 1999;55:562-571.

202. Bartha A. Proposal for subgrouping of bovine adenoviruses. Acta Vet Acad Sci Hung. 1969;19:319-321.

203. Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. Science. 1995;270:404-410.

204. Sambrook J, Fritsch, E.F., and T. Maniatis. Molecular cloning: a laboratory manual (ed 2nd Ed). Spring Harbour, N.Y.: Cold Spring Harbor Laboratory Press; 1989.

205. Zakhartchouk AN, Reddy PS, Baxi M, et al. Construction and characterization of E3-deleted bovine adenovirus type 3 expressing full-length and truncated form of bovine herpesvirus type 1 glycoprotein gD. Virology. 1998;250:220-229.

206. Evans EW, Harmon BG. A review of antimicrobial peptides: defensins and related cationic peptides. Vet Clin Pathol. 1995;24:109-116.

207. Iking-Konert C, Wagner C, Denefleh B, et al. Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease. Clin Exp Immunol. 2002;130:501-508.

208. Iking-Konert C, Cseko C, Wagner C, Stegmaier S, Andrassy K, Hansch GM. Transdifferentiation of polymorphonuclear neutrophils: acquisition of CD83 and other functional characteristics of dendritic cells. J Mol Med. 2001;79:464-474.

209. Hansch GM, Radsak M, Wagner C, et al. Expression of major histocompatibility class II antigens on polymorphonuclear neutrophils in patients with Wegener's granulomatosis. Kidney Int. 1999;55:1811-1818.

210. Okuda K, Tani K, Ishigatsubo Y, Yokota S, David CS. Antigen-pulsed neutrophils bearing Ia antigens can induce T lymphocyte proliferative response to the syngeneic or semisyngeneic antigen-primed T lymphocytes. Transplantation. 1980;30:368-372.

211. Zakhartchouk AN, Pyne C, Mutwiri GK, et al. Mucosal immunization of calves with recombinant bovine adenovirus-3: induction of protective immunity to bovine herpesvirus-1. J Gen Virol. 1999;80 (Pt 5):1263-1269.

212. Griebel PJ, Beskorwayne T, Godson DL, Popowych Y, Hein W. Cloning non-transformed sheep B cells. J Immunol Methods. 2000;237:19-28.

213. Grewal AS, Rouse BT, Babiuk LA. Mechanisms of resistant of herpesviruses: comparison of the effectiveness of different cell types in mediating antibody-dependent cell-mediated cytotoxicity. Infect Immun. 1977;15:698-703.

214. Feldman, B.V., Zinkl, J.G, Jain, N.C., Feldman, B.F., Jain, N.C., Schalm, O.W. (Eds) (2000) *Schalm's Veterinary Hematology*. 5th Ed. Lippincott Williams & Wilkins, Philadelphia.

215. Burdon RH. Temperature and animal cell protein synthesis. Symp Soc Exp Biol. 1987;41:113-133.

216. Morel O, Toti F, Hugel B, Freyssinet JM. Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. Curr Opin Hematol. 2004;11:156-164.

217. Miller JF, Kurts C, Allison J, Kosaka H, Carbone F, Heath WR. Induction of peripheral CD8+ T-cell tolerance by cross-presentation of self antigens. Immunol Rev. 1998;165:267-277.

218. Szynal M, Cleuter Y, Beskorwayne T, et al. Disruption of B-cell homeostatic control mediated by the BLV-Tax oncoprotein: association with the upregulation of Bcl-2 and signaling through NF-kappaB. Oncogene. 2003;22:4531-4542.

219. Reddy PS, Idamakanti N, Chen Y, et al. Replication-defective bovine adenovirus type 3 as an expression vector. J Virol. 1999;73:9137-9144.

220. Aich P WH, Rawlyk NA, Jalal S, Kaushik RS, Begg AA, Potter AA, Babiuk LA, Abrahamsen MS, Griebel PJ. Microarray Analysis of Gene Expression Following Preparation of Sterile Intestinal "Loops" in Calves. Canadian Journal of Animal Science. 2005;85.

221. Thoelen I, Keyaerts E, Lindberg M, Van Ranst M. Characterization of a cDNA encoding the bovine coxsackie and adenovirus receptor. Biochem Biophys Res Commun. 2001;288:805-808.

222. Los DA, Murata N. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta. 2004;1666:142-157.

223. Phadtare S. Recent developments in bacterial cold-shock response. Curr Issues Mol Biol. 2004;6:125-136.

224. Shapiro H. Practical Flow Cytometry (ed 2nd). New York; 1988.

225. Berton G, Lowell CA. Integrin signalling in neutrophils and macrophages. Cell Signal. 1999;11:621-635.

226. Akgul C, Edwards SW. Regulation of neutrophil apoptosis via death receptors. Cell Mol Life Sci. 2003;60:2402-2408.